

University of Alberta

**Regulation of carbohydrate absorption by cholecystokinin and cyclic AMP
in the rat small intestine**

by

Andrew James Hirsh



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of **Doctor of Philosophy**.

Department of **Physiology**

Edmonton, Alberta

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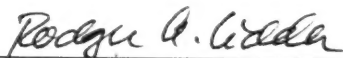
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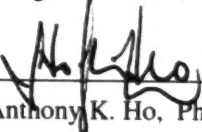
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Chris I. Cheeseman, Ph.D. - Supervisor



Rodger A. Liddle, M.D. - External Examiner



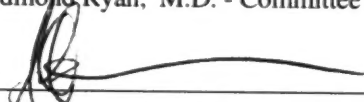
Anthony K. Ho, Ph.D. - Committee Member



Susanne C. Barton, Ph.D. - Committee Member



Edmond Ryan, M.D. - Committee Member



John J. Kennelly, Ph.D. - Committee Member

26th Nov 1997

Dedication

In memory of my father, who inspired me to be industrious in all my endeavors throughout my lifetime.

To my wife, Katherine, whose hardwork allowed me the freedom to continue my education and who endured the many years of my emotional highs and lows while performing my experiments.

And to Dr. Cheeseman, who was always very approachable and understanding towards my ideas and who made the time spent on the learning curve enjoyable.

Abstract

The small intestine's primary function is to selectively absorb nutrients and thus provide the energy required for cellular metabolism. Carbohydrate absorption is regulated by a complex set of endocrine, paracrine and neural interactions including that of cholecystokinin, a gastrointestinal peptide, which can slow carbohydrate absorption by delaying the emptying of the stomach contents into the small intestine. Also, the second messenger cAMP has been implicated in altering hexose transport rates.

The effect of CCK-8 and cAMP on the rate of hexose transport across the small intestine was examined using a dual vascular and luminal jejunal *in situ* perfusion technique. Over a physiological concentration range vascular CCK-8 reduced hexose transport in a dose-dependent manner, but had no effect on L-glucose or L-leucine absorption. In contrast, the peptide hormone had a very limited effect on streptozotocin treated animals. A specific CCK-A receptor antagonist, lorglumide abolished the effect of CCK-8, while the specific CCK-B receptor agonist CCK-4 had no effect on hexose transport. These results indicate that CCK is acting through CCK-A receptors. A lack of an effect of vascular tetrodotoxin on the action of CCK-8 suggests that these CCK-A receptors are not on intramural neurones. However, CCK may not be acting directly on the absorptive cells because a non-selective endothelin antagonist blocked the effect of vascular CCK.

At the cellular level, CCK-8 was found to reduce the abundance of the sodium dependent glucose transporter SGLT1 in the brush-border membrane, suggesting that trafficking of the transport protein produced the inhibition of hexose absorption. Analogues of cAMP and agents which elevated intracellular cAMP levels also reduced hexose absorption. However, there was no correlation between the exposure of the tissue to CCK-8 and intracellular cAMP concentrations, so it is unlikely that the hormone's actions are mediated by this signalling pathway.

These results suggest that CCK plays a physiological role in decreasing hexose absorption by acting through A-type receptors which cause the release of endothelin. Endothelin would then reduce the abundance of SGLT1 in the apical membrane. Elevated cAMP concentrations are also capable of decreasing hexose absorption, but possibly through a different mechanism from that described for CCK.

Acknowledgement

The experimental procedures that I conducted in this thesis were facilitated by the expertise of two people, Raymond Tsang and Debbie O'Neill. Ray, for teaching me the extensive surgical technique required in the dual perfusion model. And Deb, for her care and understanding when showing me the ropes with Western blotting and immunotransfer and for being a good friend. Thanks Ray and Deb. I also thank: Tom Ryan, and Dick Jones, their characters will always inspire me no matter how tough life gets, my mother for her tireless spirit and encouragement, and little Laura who continues to provide me with an infinite supply of entertainment.

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Abbreviations

• ~	Approximate
• ATP	Adenosine triphosphate
• BLM	Basolateral membrane
• BLMVs	Basolateral membrane vesicles
• BBM	Brush-border membrane
• 8-Br-cAMP	8-Bromo-3':5' cyclic adenosine monophosphate
• C	Celsius
• CCK	Cholecystokinin [*]
• CCK-4	Cholecystokinin-tetrapeptide
• CCK-8	Cholecystokinin-octapeptide
• cAMP	Adenosine 3':5'-cyclic monophosphate
• cps	Counts per second
• CHO	Chinese hamster ovary
• dpm	Decays per minute
• ET-1	Endothelin-1
• 1.0 E	Exponential
• GLUT	Glucose transporter
• [Glucose] _{int}	Intracellular glucose concentration
• GIP	Gastric inhibitory polypeptide
• GLP-2	Glucagon-like peptide 2
• g	Gram
• h	Hour
• IBMX	3-Isobutyl-1-methylxanthine
• KCN	Potassium cyanide

^{*} Experiments with CCK are the octapeptide molecular form, otherwise indicated.

• KD	Dissociation constant
• kDa	Kilodaltons
• l	Litre
• min	Minute
• mg	Milligram
• m	Milli
• M	Molar
• mol	Mole
• MSS	Membrane suspension solution
• PYY	Peptide YY
• PBS	Phosphate buffer saline
• PEG	Polyethyleneglycol
• PMSF	Phenylmethyl sulfonyl fluoride
• PBST	Phosphate buffered saline with Tween 20
• PVDF	Polyvinylidene fluoride
• RS1	Regulatory subunit 1 for SGLT1
• SGLT1	Na ⁺ -glucose cotransporter
• SS	Somatostatin
• μ	Micro
• VIP	Vasoactive intestinal peptide
• VRS	Vesicle resuspension solution

CHAPTER I

I. INTRODUCTION

A. Physiological role of the small intestine

Besides being part of the elaborate alimentary canal (mouth, esophagus, stomach, large intestine, and rectum) through which nutrients and fluids move, the small intestine provides a highly specific and organized structure where digestion is finalized and absorption of nutrients begins. The small intestine also represents an interface between the external environment and the internal milieu of the organism. It therefore must act as a selective barrier - absorbing only the useful nutrients and not allowing the entry of harmful bacteria and toxins (which may escape the stomach acids) which the organism ingests along with its food. It has been known for many years that the small intestine has the capacity to absorb all the soluble and digestible carbohydrates from the lumen, even if the concentration of the substrate is lower than the blood concentration (Barany and Sperber 1942). The fact that the lining of the gastrointestinal tract is maintained in such harsh conditions dictates a very short lifespan for the highly specialized epithelial cells.

The cell turnover rate is very rapid in the gastrointestinal tract when compared to most other cells of a multicellular organism. The whole surface layer of the gastrointestinal tract undergoes a rapid renewal process which involves the proliferation, migration, differentiation and eventual exfoliation or sloughing-off from the villus tip. Proliferation of the absorptive cells lining the mucosa takes place in the crypts (Proliferation Zone). The cells at this stage are immature proliferative cells which differentiate and mature as they migrate from the crypt onto the villus. This mass turnover rate was predicted over 100 years ago (Bizzozero 1888) and the cycling time of the absorptive cells in the small intestine has been shown to last a maximum of 57 h in rodents (King, Paterson et al. 1983; Ferraris, Villenas et al. 1992), and 5 to 6 days in humans (MacDonald, Trier et al. 1964; Shorter, Moertel et al. 1964). This enormous energy expenditure is directed at the base of the villi

found within the *crypts of Lieberkühn* which are a structural feature of the small intestine described below. Scattered amongst the numerous absorptive cells are a few endocrine cells. The endocrine cell turnover rate is not as great as that of the neighboring absorptive cells - which have no proliferative capacity (Barrett, Hobbs et al. 1995). The two other cell types (goblet and Paneth cells) have different turnover rates as well.

1. *Morphology of the small intestine*

During foetal development the gastrointestinal tract is derived from the endoderm layer, which is one of the three primitive germ layers. The nomenclature and structural properties that will be described here were taken from Fawcett (1986). The small intestine, which is the portion of the alimentary canal found between the stomach and large intestine, is a tubular structure made up of four concentric layers - the *serosal* (the outermost layer), the *muscularis* (circular and longitudinal), the *submucosal*, and the *mucosal* (the inner most layer). Of these, the mucosa is the most important regarding digestive and absorptive functions. The small intestine is grossly divisible into three unequal sized regions - the *duodenum*, *jejunum*, and *ileum*. The duodenum, beginning at the pyloric sphincter and ending at the attachment site of the ligament of Trietz, is the shortest of the three and is largely retroperitoneal and is firmly attached to the dorsal body wall. The jejunum begins at the attachment site of the ligament of Trietz and is defined as the first 40 % of the small intestine excluding the duodenum. The lower remaining 60 % is the ileum, which ends at the ileocecal valve opening into the large intestine.

As mentioned earlier one of the primary roles of the small intestine is the absorption of nutrients. To help augment the efficiency of absorption in the small intestine a number of structural specializations increase the mucosal surface area within a relatively small luminal space. Permanent crescentic folds (valves of Kerckring or *plicae circulares*) extend

half to two thirds around the lumen (**Fig. 1**). An enormous number of minute (400-600 μ M), fingerlike projections extend from the plicae circulares and throughout the mucosal lining these are known as *intestinal villi* (**Fig. 1**). The intestinal villi, which cover the entire surface of the mucosa, are more numerous in the proximal small intestine and also have a large number of projections found at the apical side called *microvilli*. The microvilli are very small hairlike projections which are only visible under an electron microscope. They help increase the surface area exposed to the lumen to an even greater extent. The surface area is also increased by invaginations called *crypts of Lieberkühn* (**Fig. 2**), which are simple tubular glands extending down to the muscularis layer (**Fig. 1**).

The surface of the mucosa is a heterogenous single layer of cells. Three different cell types can be distinguished on the surface of the villi - *absorptive* (enterocytes), *goblet*, and *endocrine* cells (**Fig. 2**). The enterocytes are the most numerous and are simple columnar epithelial cells. They are polarized with a distinct brush-border and basolateral pole. The brush-border is comprised of a surface coat representing the microvilli; it is in this area where the final stages of protein and carbohydrate digestion occur. The cells are joined together at a specific point on their lateral sides by a junctional complex consisting of - the *zonula occludens* (these are fusion points), *zonula adherens* (band of firm adhesion for terminal web), and *macula adherens*, also known as the desmosome (attachment site for cytoskeletal elements). Some of the special intracellular features of the enterocyte is that it is rich in mitochondria and has a well developed Golgi apparatus.

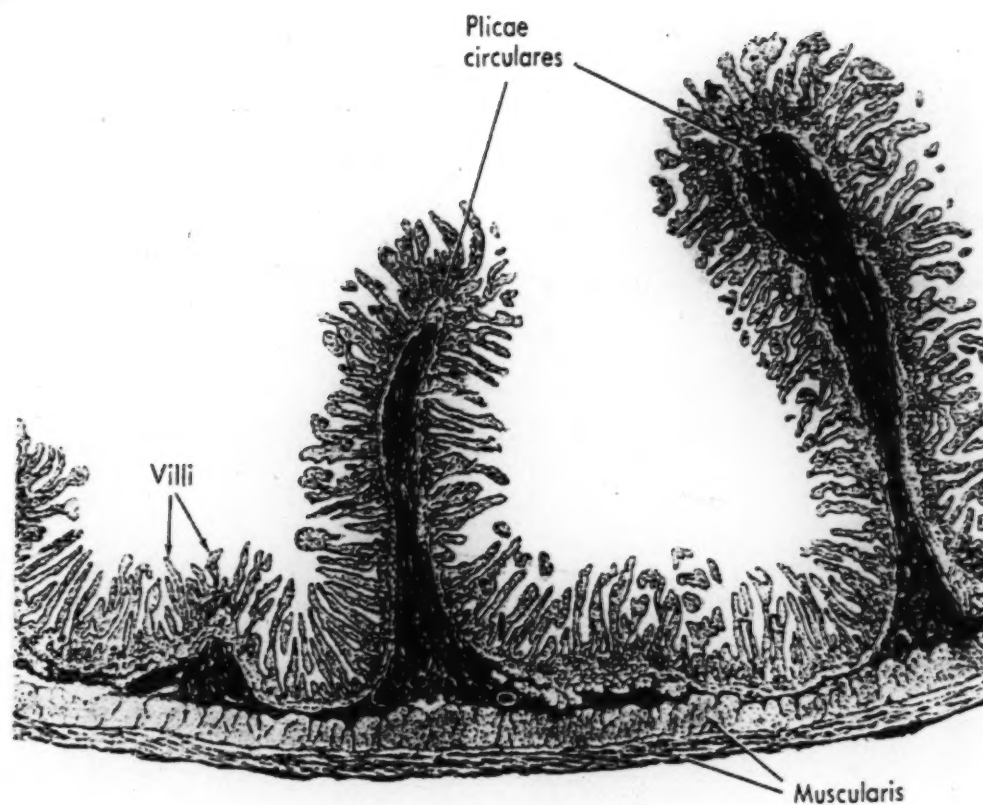


Figure 1. A drawing showing the structural specializations found in the small intestine that increase the surface area exposed to the lumen (modified from Bargmann (1962)).

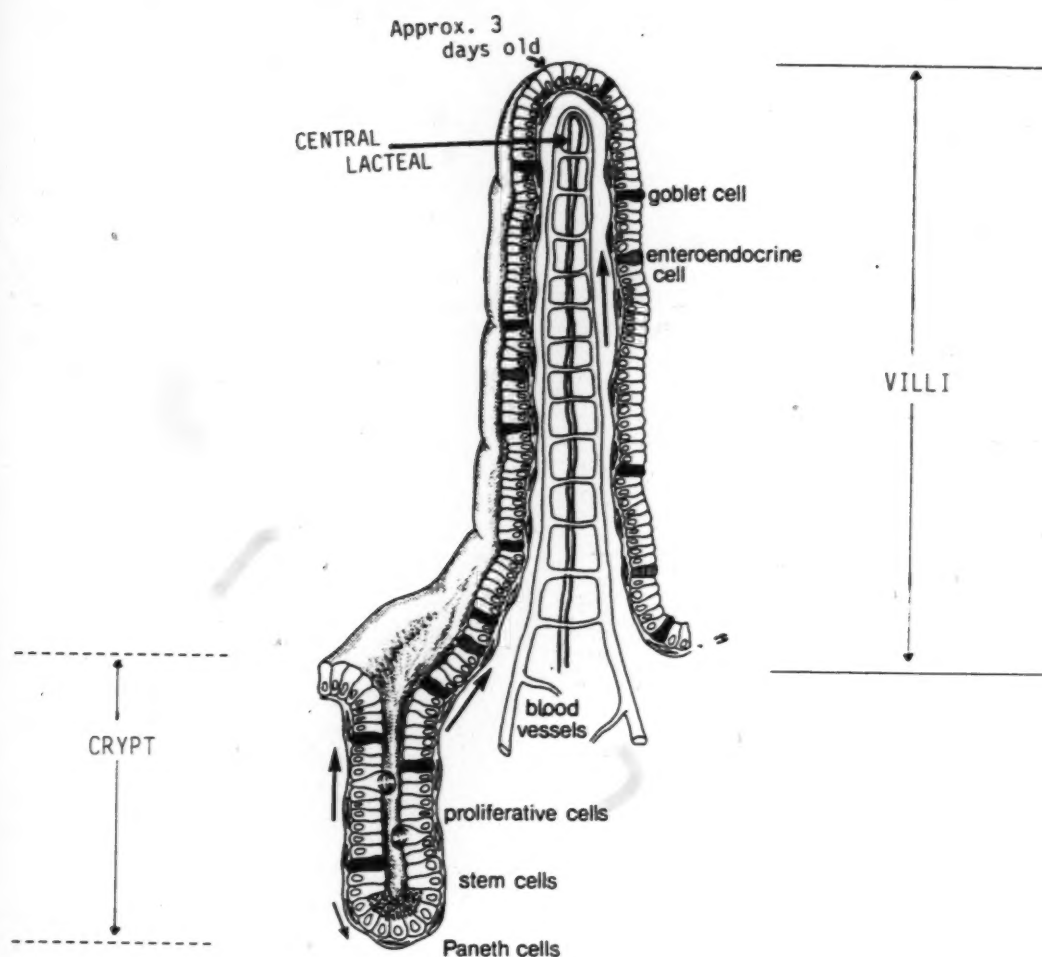


Figure 2. A schematic of the cell types, locations, and their relative abundance found lining the rat small intestine. Large vertical arrows indicate the movement of absorptive, goblet, and endocrine cells, small vertical arrows indicate the movement of Paneth cells within the crypt (modified from Potten and Loeffler (1990)).

The goblet cells are scattered irregularly among the enterocytes, and are a unicellular gland which are responsible for secreting mucus (a large glycoprotein) to lubricate and protect the surface epithelium. The endocrine cells occur singly and are scattered throughout the epithelial lining (**Fig. 2**). They have longer and thicker microvilli than the absorptive cells which suggests a possible chemoreceptor role for luminal substrates. There are many different types of endocrine cells which can be identified by the ultrastructure of their granules (Solcia, Pearse et al. 1973; Grube and Forssmann 1979). These granules synthesize and secrete peptides e.g. the I-type endocrine cell found in the proximal small intestine which synthesizes and secretes CCK. The presence of the secretory granules concentrated between the nucleus and the basal lamina suggests that their secretion products are released in the direction of the lamina propria (blood stream) and not into the lumen.

The *Paneth cell* (**Fig. 2**) found at the base of the crypts, is a secretory cell with little or no turnover (Behnke and Moe 1964) which can phagocytose bacteria (Erlandsen and Chase 1972), and secrete antimicrobial products (Ouellette and Selsted 1996).

When eating, along with the nutrients comes a large assortment of bacteria and viruses, so to protect the organism the small intestine produces a special class of antibodies immunoglobulin A and a low concentration of immunoglobulin G, that restrain bacterial proliferation, neutralize viruses and prevent penetration of enterotoxins through the mucosal lining. Macrophages, lymphocytes and plasma cells are also found in the lamina propria and are poised to protect the tissue.

The small intestine is extensively vascularized, the superior mesenteric artery branches off the descending aorta, and leads to the submucous arterial plexus, which then breaks into arterioles leading to the crypts and villi. Each villus receives one or more arterioles with an extensive capillary bed. The venous system is structurally similar to the arterial system, venules anastomose with a glandular venous plexus and then join the veins of the submucosal plexus, which leads up to the portal vein entering the liver. Lymphatic vessels are found throughout the small intestine and have a blind ending central lacteal (**Fig. 2**) at

the tip of the villus that anastomoses with the lymphatic capillaries between the crypts. The lymphatics play a specific role in fat absorption. The central lacteal in the villus receives triglycerides and cholesterol which are then delivered to the venous blood through the thoracic duct. The small intestine is innervated by preganglionic fibers from the vagus nerve and therefore is under the control of the autonomic nervous system (sympathetic, parasympathetic, and enteric). However, the enteric system can provide self contained reflex pathways which do not need input from the CNS.

B. Hexose absorption in the jejunum

As mentioned earlier the mucosal layer is the most important in regards to nutrient digestion and absorption. Similar to the liver and kidney, the small intestine has a large functional reserve. In the small intestine's case the reserve is found in its length. The majority of carbohydrates are absorbed by the jejunum, but if a load greater than the capacity of the jejunum is encountered then the ileum will absorb the remainder, thus acting as a 'functional reserve' (Booth 1961). In 1967 Booth indicated where the sites of absorption for the different nutrients occurred in the small intestine. At that time both monosaccharides and disaccharides were thought to be absorbed intact by the proximal small intestine (Booth 1967). It is now believed that only the free monosaccharides are transported across the enterocyte. To help visualize where the majority of carbohydrates are absorbed **Fig. 3** indicates carbohydrate absorption in a horizontal (along the length of the small intestine) and vertical (along the length of each villus) gradient. Even if the small intestine does not absorb the carbohydrate load the large intestine, containing a large and varied population of bacteria, will complete carbohydrate absorption using a different method from the small intestine. The colon contains bacteria which ferment the sugars producing gases (H_2 , CO_2 , CH_4) and short-chain fatty acids. The short-chain fatty acids, primarily acetate, propionate, and butyrate, are then rapidly taken up by the colonic

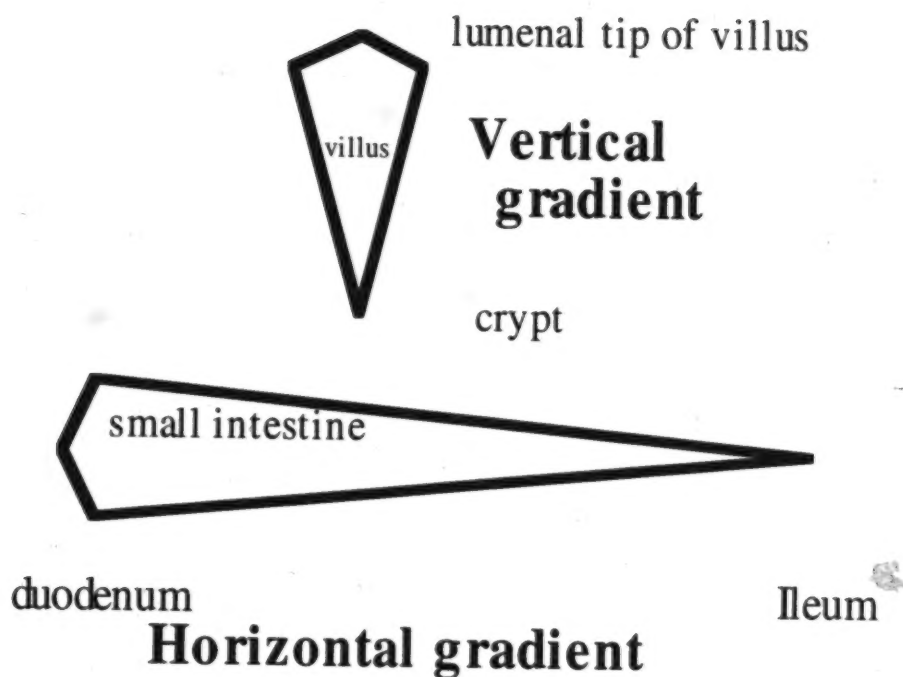


Figure 3. A schematic of carbohydrate absorption along the length of the villi and small intestine. Area of diamond indicates absorptive capacity.

mucosa (Cummings 1981). At the end of the gastrointestinal system of a normal healthy individual there should only be the insoluble and digestion resistant carbohydrates remaining in the lumen. Carbohydrate absorption across the small intestine can occur by diffusion, transepithelial transport, and a proposed paracellular solvent drag. Diffusion of sugars across the small intestine is negligible. Transepithelial transport and paracellular transport are the main mechanisms and will be discussed in the next two sections.

1. *Enterocyte hexose carrier proteins*

Six carbon sugars (hexoses) are transported from the lumen of the small intestine into the vascular bed. First, by traversing the BBM of the enterocytes and then, once in the cytosol, they exit across the BLM. Both hexoses (D-glucose and D-galactose) are transported across the BBM by the Na^+ -dependent glucose transporter (SGLT1) (Fig. 4). This transport pathway is classified as a secondary active transport mechanism, first described by Crane (1962), where the energy used to drive glucose or galactose into the cell against a concentration gradient is provided by the Na^+ electrochemical potential gradient (stoichiometry, 2 to 1, Na^+ :glucose or galactose (Kanai, Lee et al. 1994; Chen, Coady et al. 1995). This gradient is maintained by the ubiquitous Na^+/K^+ pump located on the BLM. Evidence for active transport of hexoses in the small intestine was recognized prior to the writings of Crane, (Wilson and Wiseman 1954). Some of the D-glucose, once in the cytosol, can be metabolized by the enterocyte and used as an energy source, it then exits across the BLM as lactate (Cheeseman, Shariff et al. 1994). The remaining glucose and galactose exit across the basolateral membrane by the facilitated transporter described by Kimmich and Randles (1976), characterized by Wright, van Os et al. (1980) and named GLUT2 (Davidson, Hausman et al. 1992) (Fig. 4). D-Fructose is a ketose and is one of the monosaccharides released from the hydrolysis of sucrose. It is found in the Western

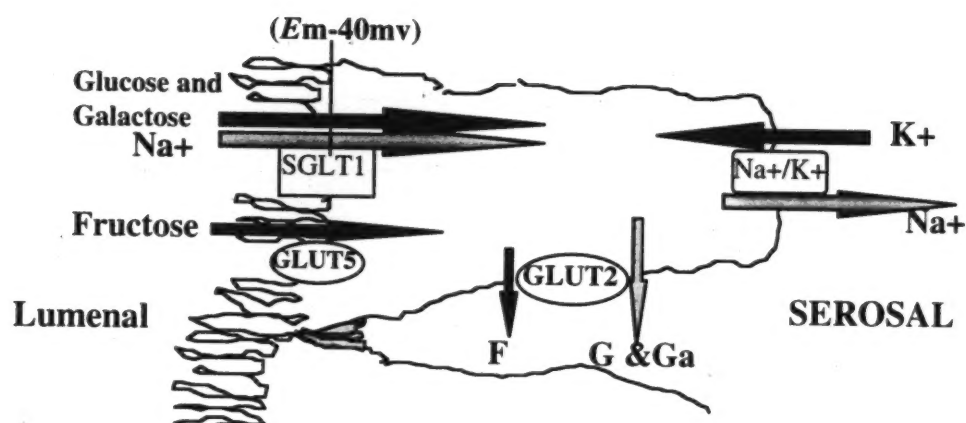


Figure 4. A schematic representing enterocytes in the small intestine showing hexose transporter locations. Arrows indicate the direction of the movement of substrate. E_m is the membrane potential, G represents glucose, Ga - galactose, and F represents fructose.

diet as an additive in many softdrinks, and fruit juices. Fructose absorption does not use the same transport carriers as glucose and galactose, but is transported across the BBM via a Na^+ -independent facilitative transporter, GLUT5 (Davidson, Hausman et al. 1992). In humans, once in the cytoplasm of the enterocyte this ketose is converted to glucose, but not in the rat which is missing the glucose-6-phosphatase enzyme (Hers and Duve 1950; Ginsburg and Hers 1960). The exit of fructose is via the same carrier as glucose and galactose, GLUT2 (Cheeseman 1993).

An understanding of the types of hexoses and their affinities for the above mentioned carrier proteins is useful when trying to identify where a change in transcellular transport rate occurs: during entry across the BBM, or exit across the BLM. To help eliminate the variable of metabolism, a non metabolizable analogue of glucose, 3-O-MG was introduced in the late 50's (Csaky and Wilson 1956). This analogue is very useful because it uses the same transport pathways as glucose (Csaky and Wilson 1956; Csaky and Glenn 1957; Csaky and Fernald 1960). It is now widely accepted as a useful substrate for monitoring absorption without interference from metabolism (Roig, Vinardell et al. 1993; Ferrer, Gil et al. 1994). Monosaccharides such as glucose have two isomers which are termed D or L. Almost all naturally occurring monosaccharides are in the D form. L-Glucose differs from D-glucose by having the hydroxyl group on the left side of the chiral carbon furthest from the first carbon on a Fischer projection. L-hexoses are very poor substrates for SGLT1, which makes them useful for determining whether the amount of diffusion and nonselective paracellular movement are occurring. Using isotopically labelled D and L isomers would therefore help to identify the preferred transport process (transepithelial or paracellular). As mentioned earlier, D-fructose maintains its structure throughout trans-enterocyte absorption in rats and is transported across the BBM by a separate Na^+ -independent facilitative carrier, and exits across the BLM by the same transporter used by the other aldoses (GLUT2). These properties therefore make it a useful hexose in identifying the location (BBM or BLM) of changes in rate of absorption.

2. *Substrate-dependent adaptation*

The ability of the intestine to change its capacity for carbohydrate absorption by an adaptive process is an area that has been intensely studied. It would be outside the scope of this thesis to address all aspects of this issue (diet, energy budgets, environmental salinity, stress, resection, starvation, and age) (Ferraris and Diamond 1997) in great detail. Therefore, I have focused on the adaptation which occurs in the absorptive capacity of the small intestine in response to changes in the diet (within hours). This is not to be confused with the subsequent section describing a rapid change (regulatory response), within minutes, in hexose absorption.

Dietary adaptation giving rise to an increased absorption of hexoses across the BBM in rodents going from a diet low in carbohydrates to one high in carbohydrates was shown to occur (Bode, Eisenhardt et al. 1981; Scharrer, Wolfram et al. 1981; Diamond 1984; Solberg 1987; Crouzoulon 1991) and also across the BLM (Cheeseman 1991). Also, when going from a high to a low carbohydrate diet a decrease in transport across the BBM (Karasov, Pond III et al. 1983; Ferraris 1992) and BLM (Cheeseman 1991) was seen. The functional consideration for the animal to adapt to these changes are two fold, 1) a caloric benefit which tends to cause the nutrient to increase its own capacity to be absorbed and 2) the biosynthetic costs involved where synthesizing and maintaining transporters tends to cause transporter expression to be repressed (Ferraris and Diamond 1997). The change in the absorptive rate of the intestine in an episodic eater (herbivore or omnivore) is thought to occur so as to match the total intestinal uptake capacity for a certain nutrient with the intake of that nutrient, plus a reserve uptake capacity of that nutrient (Ferraris 1994). Increasing the absorption by adapting to the nutrient-rich diet helps maintain the absorptive capacity at levels above prevailing intake rates; decreasing the absorption by adapting to a poor diet saves biosynthetic energy by eliminating transport capacity in excess of physiological needs

(Diamond 1991; Ferraris 1994). The adaptive response to dietary intake was shown to occur by a change in the number of transport sites per enterocyte which could result from a change in synthesis or degradation of transporters, or a change in the affinity of the transporter for its substrate (Ferraris 1994). One problem faced when determining the mechanism(s) involved in the adaptive response is the timing of the changes observed (0.5 - 3 days) (Karasov, Pond III et al. 1983; Diamond 1984). Remembering that the cell turnover rate is very rapid in this tissue, 2- 3 days in rodents, the question then becomes whether the adaptation in absorption occurs in the developing enterocyte (which is programmed with the appropriate number of transporters in the crypt according to the host's diet), or does it occur in the mature enterocytes already on the villi (Ferraris 1992)? The main mechanism(s) involved were found to occur in both the brush-border and basolateral membranes where an increase or decrease in the number of transport sites was found (Ferraris 1986; Cheeseman 1991). Also, regarding the BBM response, Ferraris showed that the crypt cells were programmed irreversibly by the signal of dietary carbohydrate (Ferraris 1992). Other factors such as levels of dietary fiber, change in caloric density of food, and starvation may also modify this adaptive response.

3. *Acute regulation of hexose absorption*

Rapid regulation of hexose transport has been shown to occur with the facilitative glucose transporters. The majority of studies indicate the isoforms GLUT1 and GLUT4 transporters can be upregulated by insulin, by a mechanism which was first described by Cushman and Wardzala (1980), and later confirmed by James, Brown et al. (1988). This regulation, which causes a change in the rate of hexose transport occurs within minutes. The mechanism involved is a translocation phenomenon by which transporters are inserted into the plasma membrane from a storage pool found within the cytoplasm. This rapid translocation regulation is found not only with the facilitative hexose carriers, but also with

insulin-like growth factor II (Oka, Rozek et al. 1985), transferrin (Davis, Corvera et al. 1986), and secretory carrier-associated membrane proteins (Laurie, Cain et al. 1993).

The timing of the physiological event is what primarily classifies it as an adaptive or regulatory response. The techniques used in this thesis dictate that the event monitored will be an acute regulatory response. Much work has been done identifying adaptive changes associated with hexose absorption in the small intestine and only recently have some studies been completed showing rapid (within 1 h) regulation of hexose absorption in the small intestine (Debnam 1994; Pennington, Corpe et al. 1994; Hardin, Wong et al. 1996; Cheeseman 1997). Interestingly, most of the signalling involved in the rapid regulation was via endogenously produced peptides. To date, the mechanism(s) of this acute regulation in the small intestine has not been investigated.

4. *Paracellular movement*

As mentioned earlier, absorption of molecules across the small intestine may also occur in part by a paracellular route. This pathway warrants discussion due to its possible significance during experimentation. The paracellular movement theory was introduced by Pappenheimer and Reiss (1987) to help explain how, under certain experimental conditions, 1. The kinetics of hexose carrier proteins which are used for transcellular transport are not able to accommodate the amount of glucose found in the circulation after supraphysiological concentrations of glucose were administered to the lumen of the intestine (Pappenheimer 1993); 2. certain hydrophilic low and high molecular weight compounds, as well as oligopeptides (Atisook and Madara 1991), which are not actively transported, can get into the urine of animals when administered with high doses of glucose (Pappenheimer 1990).

This movement is proposed to occur by a nonselective solvent drag through intercellular spaces. The nutrients would pass through the zonula occludens (tight junction) which is

regulated by the activity of the sodium-coupled transporter in the BBM (Madara and Pappenheimer 1987). The regulation of the tight junction is modulated by cytoskeletal elements which dilate the tight junctions found in the terminal web of all enterocytes. The importance of this absorptive route in the small intestine has been challenged by groups who measured glucose absorption directly in the rodent (Schwartz, Furne et al. 1995; Hirsh, Tsang et al. 1996), as well as in humans (Fine, Santa Ana et al. 1993). However, *in vitro* experiments using isolated segments (Ussing chambers) apparently show paracellular movement playing a major role in the small intestine (Perez 1997).

My concerns regarding this route are: **1)** the selective barrier function of the small intestine becomes invalid, (selectivity being determined only by the size of the pore) **2)** the luminal glucose concentrations required to effectively initiate the required solvent drag far exceed the physiological concentrations found in the lumen (Ferraris, Yasharpour et al. 1990), **3)** the driving force (solvent drag) and direction is not adequately explained or described, and finally no mention that other routes of transport, such as endocytosis (Olmsted, Dunny et al. 1994) or diffusion through molecular volume sensitive aqueous pores (Hamilton, Rothwell et al. 1987), could be responsible for the observed absorption.

Interest in the regulation of the zonula occludens has been generated by the paracellular movement theory. Cultured enterocyte-like cells have been shown to modify their tight junctions by reducing ATP levels using nitrous oxide (Salzman, Menconi et al. 1995), increasing cAMP (Duffey, Hainau et al. 1981), increasing PKC (Stenson, Easom et al. 1993), and by enterotoxins produced by the bacterial flora (Fasano and Uzzau 1997; Obiso, Azghani et al. 1997). Whether these regulatory pathways are involved *in vivo* is still being evaluated.

5. *Post-absorptive fate of glucose*

The first law of thermodynamics states simply that 'energy can be changed from one form to another, but it cannot be created or destroyed'. Carbohydrates are the primary energy source for all living cells. Entering into the host from the diet a major source of carbohydrate comes from starch, disaccharides and glycogen. These carbohydrates cannot be transcellularly absorbed by the small intestine and are therefore not useful to the host unless hydrolyzed to the simple form (monosaccharides). The hydrolysis of the complex carbohydrates occurs by enzymes found in the salivary and pancreatic secretions and on the mucosal lining of the small intestine. Hydrolysis of complex carbohydrates occurs at the glycosidic bonds (α 1-4, 1-6, and β 1-4) of the dietary poly- and disaccharides to form primarily monosaccharides: D-glucose, D-galactose and D-fructose. L-hexoses are not commonly found in the chyme and are poor substrates for transcellular absorption. After absorption by the small intestine, these monosaccharides are used by all cells as their primary source of energy, producing end-products such as ATP, CO_2 and H_2O . The circulating level of glucose is maintained within a fairly narrow concentration range even though the dietary intake is episodic, therefore a balance exists between glucose entering and leaving the system (**Fig. 5**). The free glucose plasma concentration is primarily maintained by dietary intake, the rate of uptake into cells, and the glucostatic action of the liver. Before discussing the glucostatic action of the liver I will first briefly discuss the catabolic fate of glucose in a cell.

Once glucose enters the cell by one of the glucose transporters it normally undergoes glycolysis. Briefly, glycolysis is the splitting of glucose (a hexose) by multiple enzymes found in the cytoplasm, forming pyruvic acid (two triose), and a small amount of ATP and NADH. The pyruvic acid is converted to either lactic acid, anaerobically, or aerobically in the mitochondria through the Krebs cycle and electron transport chain to form ATP, with CO_2 , and H_2O as by-products.

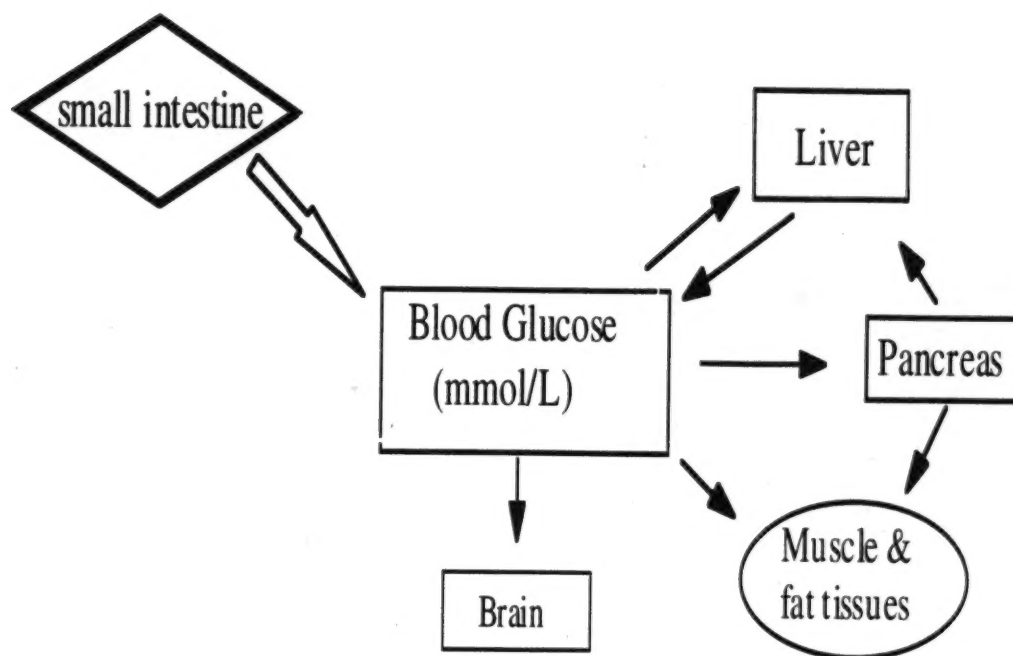


Figure 5. A basic schematic showing movement of free glucose and the main organs and tissues indicating their contribution to the plasma glucose concentrations. Arrows leading out of the pancreas indicate where hormones are acting.

The liver is in an ideal location for modulating the plasma glucose concentration as it receives venous blood coming from both the intestinal tract and the pancreas. When the supply exceeds the rate of utilization of glucose, the surplus is converted in the hepatocytes into glycogen, an insoluble, fairly osmotically inactive polysaccharide, primarily via a hormone sensitive (insulin: glycogen, ratio dependant) pathway. If utilization exceeds supply the opposite occurs: glycogen is converted back to glucose and transported into the circulation. This is a key feature since the supply of carbohydrate is not continuous and metabolism is ongoing and can fluctuate at different times in various tissues of the animal. It should be noted that fats and proteins, are also broken down, absorbed and converted back to larger complexes, which emphasizes the importance of the intestine as a selective barrier. The hormonal regulation of the liver is mediated primarily by insulin and glucagon, which are synthesized and secreted by the glucose-sensitive pancreatic islet cells. During absorption, the blood glucose concentration increases, changing intracellular metabolism which then promotes the release of insulin by this shift in metabolism in the B cell. Insulin acts on the liver to decrease glucose output through increased glycogen synthesis by increasing glycogen synthase and decreasing gluconeogenesis (Ortmeyer, Bodkin et al. 1997). Glucagon acts in the opposite direction, it stimulates glucose - 6 phosphatase to synthesize more glucose from the stored glycogen and it promotes gluconeogenesis and inhibits fatty acid synthesis.

This section is a simplified general review of the systems and pathways involved in carbohydrate metabolism in an organism and by no means should it be considered as a comprehensive review.

C. The vascular and lumenally perfused intestinal preparation

A major problem associated with accurately measuring substrate absorption and accumulation in isolated segments of tissue is the poor tissue penetration of substances

added to the perfusate which leads to nonphysiological concentrations and possibly spurious results. One other problem using isolated segments *in vitro* is the effects on the transporters which operate according to a concentration gradient (such as GLUT2). Without a vascular circuit, the enterocytes accumulate hexoses within themselves, a situation which apparently occurs little, if at all, *in vivo* (Bronk and Ingham 1979). A technique which circumvents this problem is the dual lumenal and vascular perfused *in situ* preparation. This technique maintains the vascular circuit of a segment of small intestine and permits control of a substance (peptide) concentration within a physiological range (Dubois and Roy 1969; Hanson and Parsons 1976). This technique has been used by a number of investigators Hanson and Parsons (1976); Bronk and Ingham (1979); Cheeseman (1981); Miyazaki, Sunada et al. (1986); Roig, Vinardell et al. (1993); and Pennington, Corpe et al. (1994), and has proven useful in monitoring rapid changes that occur in hexose absorption induced by physiological concentrations of insulin (Pennington, Corpe et al. 1994). The technique is also very practical for isolating lumenal signals from vascular ones e.g. peptides released from enteroendocrine cells found throughout the small intestine (Wojcikowski, Maier et al. 1985; Dakka, Cuber et al. 1993). The principle of this method is to measure the appearance of isotopically labelled hexose(s) in the vascular effluent when initially added to the lumenal perfusate.

Because this dual perfusion preparation of the small intestine does not disrupt the normal tissue geometry some of the potential interactions between neural as well as endocrine tissue on hexose absorption will also be addressed.

D. Cholecystokinin and nutrient assimilation

As mentioned earlier the mucosa of the small intestine contains a heterogenous population of cells. The cells that are responsible for the synthesis and secretion of CCK in

the intestinal mucosa are I-type enteroendocrine cells* (using the Wiesbaden classification (Solcia, Pearse et al. 1973)) which are found predominantly in the proximal small intestine.

After a meal is ingested, the nutrients reaching the small intestine cause a rise in plasma CCK levels which, in turn, can simultaneously cause a delay in gastric emptying, an increased bile secretion via its effects on the gallbladder and sphincter of Oddi, and an increase in the secretory products from the pancreas. The aim of this section is to inform the reader about the history of CCK and its involvement in glucose homeostasis, by reviewing selected articles on two of the physiological events which have an effect on plasma glucose concentration (gastric emptying and pancreatic secretions). The articles reviewed date back to the early 1900's and were chosen for their use of either human or rodent models, employing both *in vivo* and *in vitro* techniques, although much of the early work on gastric emptying was performed on the canine model.

1. History of CCK

Secretin was the name given to the first peptide extracted from the mucosa of the small intestine. When infused intravenously it increased the secretory products from the pancreas and enhanced bile flow from the gallbladder (Bayliss and Starling 1902). It was suggested that the exogenous administration of secretin, stimulated emptying of the gallbladder which then increased the bile flow (Braga and Campos 1919). Discussion following from this work questioned whether the secretin-induced physiological responses were mediated by a receptor binding phenomenon or an indirect neuromodulatory action. The work by Ivy and Oldberg (1928) examined these questions by showing that changes in intragallbladder pressure were not inhibited by atropine when secretin was applied to the system in both a canine and feline model. The same group found that when highly purified extracts of secretin were administered there was no effect on gallbladder pressure. This work

* Described by Heidenhain in 1870.

therefore suggested that another substance, closely related to secretin, was causing the change in muscular tone of the gallbladder. One year later the same group performed cross-circulation experiments on dogs. They found that when the duodenum of the donor was stimulated with diluted HCl both 'recipient' as well as 'donor' produced gallbladder contractions. This indicated that HCl could stimulate the release of a hormone from the duodenum and simultaneously cause the gallbladder contraction (Ivy and Oldberg 1928). From these experiment the hormone was given the name "Cholecystokinin, (CCK)" after the Greek terms 'Chole' - bile; 'Cysto' - bladder; 'Kinin' - contraction (Ivy and Oldberg 1928). Harper and Raper (1943) thought they had discovered a new peptide, which they named 'pancreozymin'. The distribution was found to be in the same regions as secretin and it was shown to increase pancreatic juice secretion. It was only after 20 years of purifying gastrointestinal peptides that both CCK and pancreozymin, which stimulate the pancreas and cause the expulsion of bile, respectively were determined to be one and the same peptide. At that time it was given the name Cholecystokinin-Pancreozymin (CCK-PZ) (Jorpes and Mutt 1966) but it now goes by simply CCK (Mutt and Jorpes 1968). CCK is synthesized and secreted by I-type cells, which are scattered throughout the duodenum and jejunal mucosa of dogs (Polak, Bloom et al. 1975), humans (Buffa, Solcia et al. 1976), and rats (Eng, Du et al. 1984), and it has also been localized in the brain of sheep (Dockray, Gregory et al. 1978) and humans (Rehfeld 1978; Miller, Jardine et al. 1984). The fact that CCK is found in the endocrine cells of the gastrointestinal tract as well as in the CNS makes CCK a hormone as well as a neurotransmitter (Williams 1982).

Originally, CCK was purified from pig small intestines as a 33-amino acid peptide (Mutt and Jorpes 1968). Since then, molecular forms ranging from 5 to 83 amino acid residues have been isolated (Reeve JR, Eysselein et al. 1986; Eberlein, Eysselein et al. 1987; Eberlein, Eysselein et al. 1992). The COOH-terminal amino acid sequence of CCK '-Met-Asp-Phe- NH₂' described by Mutt and Jorpes (1968), was found to be identical to the corresponding sequence of gastrin, but CCK belongs to a group of naturally occurring

polypeptides with a COOH-terminal α amide structure instead of a free COOH-terminal carboxyl group. The structure-activity relationship for CCK indicates that to have specific receptor binding an unusual sulfation of the tyrosine residue, position 7, was essential for strong activity (Williams 1982; Vinayek, Jensen et al. 1987), and that the strong acidic charge and its position in relationship to the peptide backbone is what maintains the biological activity (Bodanšky, Natarajan et al. 1978).

A single gene is responsible for encoding CCK in all species; the gene location is on chromosome 3 in humans (Lund, GeurtsvanKessel et al. 1986; Takahashi, Fukushima et al. 1986), and gene expression was shown not to parallel increased secretions of CCK, as exhibited using bombesin (Kanayama and Liddle 1991). The gene consists of ~ 7 kilobases with 3 exons. The first exon encodes the 5' untranslated portion of CCK mRNA, the second contains sequences for encoding the signal peptide and prohormone regions, and the third, encodes the biologically important region of the peptide (Deschenes et al. 1985). Also, the transcription from the gene yields an mRNA of approximately 750 bases of which only 345 encode protein, and CCK is translated to prepro-CCK which is a 115 amino acid polypeptide. The major molecular form found in most species is CCK-58 (Eyesselein et al. 1990) but intracellular processing can generate, by cleavage at the mono or dibasic residues, the smaller biologically active peptides (Reeve et al. 1994). Also the concentration of protein or carbohydrate has been suggested to cause a shift in molecular forms of CCK release (Eberlein et al. 1988).

The ability to measure free plasma concentration of CCK gave rise to an enormous amount of pertinent information. 1) Identifying possible signaling pathways involved in CCK secretion, 2) identify the timing of release and assessment of viability in the systemic circulation, and 3) identify concentration boundaries for CCK (Liddle, Goldfine et al. 1984; Liddle, Morita et al. 1986). The endogenous free plasma concentrations in fasted rats and humans, is 0.5 - 1 picomolar and it increases to approximately 8 pM in humans fed a liquid meal (Liddle, Goldfine et al. 1985), and approximately 6 pM in rats fed a liquid

meal (Liddle, Goldfine et al. 1984). The human and rat CCK plasma concentrations were both determined using the same CCK bioassay which is a quantitative measure of amylase secretion from isolated rat pancreatic acinar cells. The concentration of CCK increased to a maximum at approximately 45 minutes after the liquid meal, and then declined to a slightly elevated level lasting 3-5 hours (Fig. 6). From these studies a possible signalling pathway for CCK release was defined: ingestion of food (liquid meal) causes an increase in plasma CCK levels in humans and in rats. The characterization of the mechanisms of how nutrients stimulate CCK release is still ongoing. A feedback mechanism has been suggested involving proteases in which inactive proteases stimulate the release and active ones inhibit it. Also, three naturally occurring CCK-releasing factors have been suggested to regulate the release: monitor peptide, which is produced by the pancreas and secreted as part of the pancreatic juice (Iwai, Fukuoka et al. 1986), luminal CCK-releasing factors (LCRF), and diazapam-binding inhibitor (DBI) which are both secreted by the intestine into the lumen during basal conditions initially described by Lu et al. (1989) and characterized by Herzig, Schon et al. (1996); Spannagel, Green et al. (1996). All of these factors are likely to play a role in regulating CCK plasma levels, at the time of digestion and during fasted states.

The intracellular signaling involved in CCK release includes multiple pathways: activation of adenylyl cyclase, and increasing intracellular calcium (Barber, Walsh et al. 1986; Koop and Buchan 1992; Sharara, Bouras et al. 1993; Prpic, Basavappa et al. 1994). CCK secretion increased when cAMP analogues and forskolin were added to isolated intestinal cells (Barber, Walsh et al. 1986; Koop and Buchan 1992); also CCK secretion increased when a non-specific phosphodiesterase inhibitor (IBMX) was added to a cell culture line which is known to secrete CCK (STC-1 cells) (Prpic, Basavappa et al. 1994).

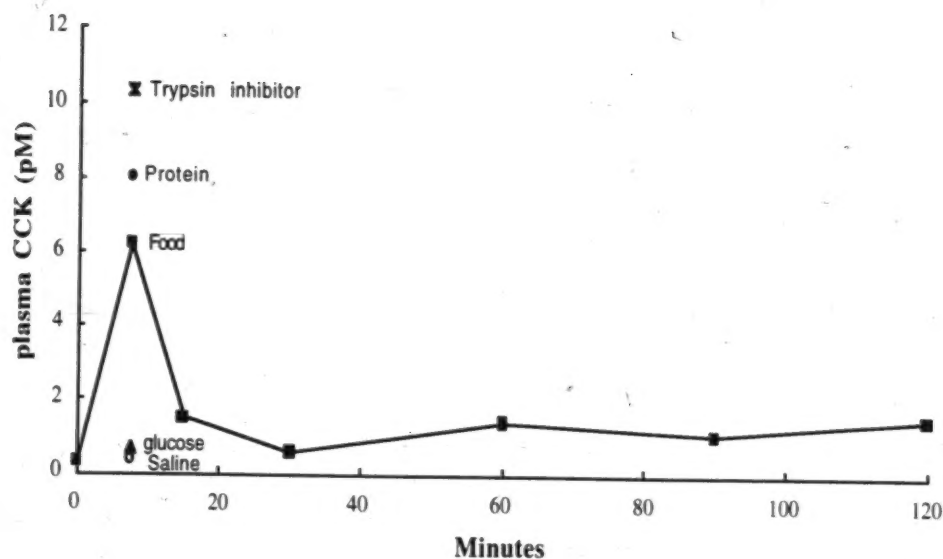


Figure 6. The effect of food, trypsin inhibitor, protein or glucose on plasma CCK levels in rats (modified from Liddle *et al* 1984, and Sharara *et al.* 1993). Food represents a liquid meal (5 ml) of Ensure Plus, protein represents 18% casein. The trypsin inhibitor is to provide evidence for trypsin inactivation pathway.

2. CCK receptors

There are three classes of receptors that bind CCK: 1) the well characterized CCK-A-type which was initially characterized by cDNA cloning after purifying the receptor protein from rat pancreas (Wank, Harkins et al. 1992), 2) the CCK-B-type, which was cloned from canine gastric parietal cells (Kopin, Lee et al. 1992) and was shown to be 50 % homologous to the rat CCK-A, with similar affinity for both CCK and gastrin; and 3), a less well defined gastrin-CCK-C receptor, described for some cell lines, which has not yet been cloned (Seva, Dickinson et al. 1994; Singh, Owlia et al. 1995). The CCK-A receptor was characterized by cDNA cloning, the predicted protein product was a typical 7-transmembrane receptor containing 444 amino acid residues with a KD of 50, which was determined after purification of receptor protein in the rat pancreas (Wank, Harkins et al. 1992). The A-type receptor was named for its alimentary origin, whereas the B-type was found primarily in the brain. The A-type was shown to have a 1000-fold higher affinity for sulfated CCK than for gastrin in the sulfated or nonsulfated form (Jensen, Lemp et al. 1980; Steigerwalt and Williams 1981). The CCK-A type receptor has been characterized in the rat acinar pancreatic cell (Rosenzweig, Miller et al. 1983), bovine gallbladder (Steigerwalt, Goldfine et al. 1984), lower esophageal sphincter (Rattan and Goyal 1986), gastric somatostatin cells (Lloyd, Maxwell et al. 1994), and in the distal intestine (ileum and colon, Chang and Lotti 1986). The CCK-B type receptor has been characterized in the cerebral cortex of mice (Steigerwalt and Williams 1981).

3. Physiological actions of CCK on free plasma glucose concentrations

Cholecystokinin has numerous physiological effects related to nutrient assimilation in an organism: causing contraction of the gallbladder and relaxation of the sphincter of Oddi, inhibiting acid secretion in the stomach, increasing exocrine pancreatic secretion, and

regulating pancreatic growth. Included with these effects CCK has been shown to regulate free plasma glucose concentrations through its actions on gastric emptying, endocrine pancreatic secretion, and cessation of food intake (Gibbs, Young et al. 1973; Lieverse, Jansen et al. 1994). Two other actions of CCK which are specific to the intestine are a decrease in transit time in the small intestine (Bertaccini and Agosti 1971; Thor, Laskiewicz et al. 1988; Wang, Soltesz et al. 1996) and an increase in mucosal anion secretion (Kachur, Phillips et al. 1991; Kachur, Wang et al. 1991). The next three sections will be devoted to outlining the role of CCK on glucose homeostasis through three pathways: causing a delay in nutrient delivery to the small intestine, increasing the amount of digestive enzymes and monitor peptide from the pancreas, and finally how it is involved in insulin secretion from the pancreas.

a) Gastric emptying and the influence of CCK

Emptying of the stomach is normally promoted by relaxation of the organ and by peristaltic waves generated in the antrum. The pyloric sphincter does not play a large role in gastric emptying. There is an enterogastric pathway which inhibits gastric emptying, it is activated when chyme enters the duodenum promoting both the antral peristalsis and relaxation of the pyloric sphincter. Inhibition of gastric emptying also occurs by hormonal feedback, originating from the duodenum and jejunum. How this was determined will be discussed below.

Early work by Sokolov (1904), using the canine model, showed that when acid came into contact with duodenal mucosa it caused an inhibition of gastric acid secretion. This early experiment prompted a fundamental question - what physiological process could be involved in this inhibitory response? Gillespie and Grossman (1964) showed, using denervated fundic pouches from dogs, that CCK given intravenously would inhibit gastric acid secretion. Johnson, Brown et al. (1966) added to this work by showing that

administered CCK caused gastric motor activity (gastric pressure, frequency and amplitude of contractions of stomach) to decline in humans. The rate at which the stomach empties was not addressed at that time.

The rate at which the stomach empties was shown to be regulated by the volume and energy content of a meal (Hunt and Stubbs 1975). The discovery of the actions of CCK on inhibiting the rate of emptying followed soon after (Debas, Farooq et al. 1975). However, no mechanism of action was discussed as to how CCK delayed food from reaching the small intestine. A few other intrinsic properties in the ingested food could affect the rate of gastric emptying (pH, osmolarity, ion and nutrient content, and temperature) (Barker, Cochran et al. 1978; Bateman 1982; Hunt 1984). As mentioned earlier, (Liddle, Goldfine et al. 1984) developed a bioassay to measure plasma CCK concentrations. The timing and signaling of its release strongly supported a regulatory role in gastric motility. This led to the hypothesis of a feedback loop, whereby CCK would regulate its own release - fewer nutrients in the small intestine, less CCK! The first physiological link between CCK release and a delay in gastric emptying was shown by Liddle, Morita et al. (1986). Ingestion of a liquid meal caused a rise in plasma CCK levels along with a delay in emptying, which was reproduced by infusing exogenous CCK-8 intravenously in humans. A possible mechanism of action for the delay was not addressed.

One of the first articles that indicated a neuromodulating effect of CCK on gastric emptying was from the work by Raybould, Roberts et al. (1987). Intravenous administration of CCK-8 (0.3-33 pM) caused a decrease in intragastric pressure by pathways involving the vagal and splanchnic nerves: vagally by a nonadrenergic pathway, and by an α -adrenergic pathway acting on the splanchnic nerve in rats *in vivo*. One question that had to be addressed was - what was the physiological reason for delaying the arrival of all nutrients to the small intestine? Liddle, Rushakoff et al. (1988) suggested that the CCK-induced delay is instrumental in reducing postprandial hyperglycemia. Humans, given an oral liquid glucose load with and without an intravenous infusion of CCK,

showed significant changes in plasma glucose, CCK, and insulin concentrations (Liddle, Rushakoff et al. 1988). When CCK was infused, CCK levels increased concomitantly with a delay in gastric emptying, reduced insulin level, and a significant decrease in glucose concentration (Liddle, Rushakoff et al. 1988).

The advent of CCK antagonists in the late 1980's confounded the issue of CCK action on gastric emptying. To strengthen CCK's role in glucose homeostasis through delaying gastric emptying, Liddle, Gertz et al. (1989), using similar techniques to Liddle, Rushakoff et al. (1988), including gallbladder contractility as an internal control. They showed that MK 329, a CCK-A type antagonist, when given orally, inhibited CCK-induced gallbladder contractility and increased plasma CCK levels, but had no effect on gastric emptying. They concluded that "other factors" compensate for, or obscured the effect of, CCK in this experiment. Fried, Erlacher et al. (1991) added to the dilemma by showing that loxiglumide (a more specific CCK-A receptor antagonist) when given to individuals prior to a liquid meal, both accelerated gastric emptying per se without CCK and abolished the inhibitory effect of CCK on delaying emptying. The same conclusions were made in later human studies using the CCK antagonist loxiglumide (Konturek, Thor et al. 1994; Borovicka, Kreiss et al. 1996). Most of the work to date on humans has been focused on cause and effect. To explore the mechanism of CCK-induced inhibition of gastric emptying, which could possibly answer the conflicting results using the antagonists in human studies, Lotti, Pendleton et al. (1987) found that CCK's actions were blocked using MK-329 (L-364,718) in rats *in vivo*. Two articles by Forster et al., (1990, 1991) showed that the action of endogenously produced CCK following a meal rich in protein was suppressed by a vasoactive intestinal peptide (VIP) antibody in the rat. The action appears to be mediated through the primary afferent fibres (mechanoreceptor mediated) which work in parallel with an inhibitory vago-vagal reflex which regulates VIP neurons in the body of the stomach, thus delaying gastric emptying (Forster, Green et al. 1990; Forster, Green et al. 1991). This mechanism was supported by Widdop, Krstew et al. (1994).

From these studies linking CCK and gastric emptying I conclude that CCK action, in rats, is localized on the primary afferent fibres (mechanoreceptor mediated) which work in parallel with an inhibitory vago-vagal reflex, regulating VIP neurons in the body of the stomach leading to a delay in gastric emptying. In humans, the CCK mechanism involving gastric emptying is not clear due to conflicting results with CCK antagonist, and require more studies.

b) CCK and its Role in Pancreatic Secretion

The pancreas is a mixture of exocrine and endocrine tissues. Its function is to secrete digestive enzymes, protease inhibitors and signaling peptides as well as aqueous alkaline fluid into the duodenum. The enzymes are essential for proper digestion of nutrients and thus facilitate intestinal absorption, and the alkaline fluid helps buffer the pH of the chyme leaving the stomach. The endocrine portion consists of islets of Langerhans which synthesize and secrete hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide) into the blood stream. CCK has been shown to regulate exocrine pancreatic secretion and to regulate one of the positive feedback mechanisms (the trypsin sensitive monitor peptide, which is an effective signal for CCK release), and it has been suggested to act as an incretin in certain species.

(1) Endocrine function and CCK

Cholecystokinin, also known as Pancreozymin, was studied for its physiological effect of increasing pancreatic juice secretion. Meade, Kneubuhler et al. (1967), examined the effects of CCK on the endocrine portion of the pancreas where they showed that CCK could increase the secretion of insulin in dogs. Dupre, Curtis et al. (1969) followed this study and confirmed that CCK increased insulin secretion in humans; this effect was seen following an infusion of CCK itself or following an infusion of arginine (which increases

endogenous CCK release). This new role of CCK as a possible incretin, was challenged by the work of Buchanan, Vance et al. (1969). They showed that exogenous CCK did not stimulate secretion in isolated islets from cats, and therefore suggested a possible 'neural component of indirect action,' or that there are distinct species variations. Fussganger, Straub et al. (1969), using isolated perfused pancreas preparations from the rat found that CCK could increase the secretion of insulin, and also cause a slight increase in glucagon secretion.

The variations seen in these earlier studies possibly result from sample contamination, e.g. with gastric inhibitory peptide, which would lead to spurious conclusions about CCK's action on insulin secretion. The arrival of synthetic CCK (octapeptide) was useful in eliminating any possible contamination as an explanation for variation in the results. The use of intravenous synthetic peptide caused an increase in insulin secretion in dogs, *in vivo*, (Frame, Davidson et al. 1975), and *in vitro*, (Ipp, Dobbs et al. 1977). Also, Otsuki, Sakamoto et al. (1979), using isolated perfused pancreas from the rat, showed that CCK elicits insulin and glucagon release in addition to stimulating the flow rate of pancreatic juice. No cytotropic function of CCK was mentioned. Sakamoto, Otsuki et al. (1982) completed studies on isolated perfused rat pancreas and measured both the amylase output and insulin secretion. They found that synthetic CCK-8, infused at lower concentrations than those which were currently being used, would significantly and coincidentally increase secretion from both tissue types, therefore contributing to the 'entero-insular axis' which was suggested by Pederson and Brown (1978). Verspohl, Ammon et al. (1986) found that CCK could directly stimulate insulin release from isolated islets of Langerhans from rats, and suggested a possible direct receptor ligand binding. This was supported by Sakamoto, Goldfine et al. (1985) who showed binding of CCK to CCK-type receptors on the β cells in rats. Furthermore, Funakoshi, Miyasaka et al. (1996) showed a dysfunctional insulin secretion in rats not expressing the CCK-A type receptors. The action of the CCK-A type antagonist loxiglumide inhibited phosphatidyl inositol hydrolysis within the β cell in rat

pancreas, which is the major pathway involved in CCK-induced insulin secretion (Karlsson and Ahren 1991). A novel pathway was described by Kelley, Zawulich et al. (1994), showing the action of CCK binding to its specific receptor causes stimulation of phospholipase C within the β cell, but the CCK activation had to be in unison with glucose metabolism to allow the translocation of protein kinase C which would result in the release of insulin. This pathway was supported by Babb, Tarpley et al. (1996), who demonstrated that CCK potentiates a glucose-induced insulin secretion. Kogire, Gomez et al. (1992) disagreed about the effects of CCK. They found no effect on insulin release when exogenous CCK was administered to rats, but mentioned that there is a possible down-regulation of the CCK receptors (Roettger, Rentsch et al. 1995) on the β cell caused by exogenous CCK introduction.

In humans the incretin action of CCK was not as obvious. Rushakoff, Goldfine et al. (1987) found that monitoring postprandial CCK levels in humans and infusing similar exogenous concentrations of CCK showed that CCK potentiates the effects of amino acid-induced insulin secretion. This work was challenged by Fieseler, Bridenbaugh et al. (1995) who indicated that CCK did not potentiate amino acid-induced insulin secretion, whereas GIP and GLP-1 did. This is similar to the dilemma found with gastric emptying Schwarzendrube, Niederau et al. (1991) who found that loxiglumide failed to alter circulating insulin and C-peptide levels caused by high plasma glucose (after ingestion of a liquid meal). Yet, loxiglumide had a substantial effect on other CCK-influenced physiological responses therefore, CCK's action was doubtful as a possible incretin. Baum, Nauck et al. (1992) also added that 'there is no direct effect of CCK on β cells in humans,' and 'the effects of loxiglumide are possibly indirect via an action on the exocrine secretion'. These studies indicate that there is species variation regarding the action of CCK on insulin release, in humans it does not act as an incretin and in rats it does.

(2) Exocrine secretion and CCK

Cholecystokinin's effect on the exocrine tissue of the pancreas has been extensively studied in the past and present. Membrane receptor characterization, interaction of G protein, and signal transduction mechanisms, were all characterized using an isolated pancreatic acinar cell preparation developed by Williams, Korc et al. (1978).

In the early 1900's I.P. Pavlov showed that by acidifying the upper intestine of dogs, the pancreatic juice would have a low protein content; whereas, when the animal was vagally stimulated the pancreatic juice would have a rich protein content. From these experiments two hypothesis were postulated for increased pancreatic secretion, 1., a substance (?) is released in response to the stimulus, or 2., that innervation would regulate pancreatic enzyme secretions. It was not until 1943 that Harper and Raper, doing experiments with the proposed 'Pancreozymin' established that an extract from the upper intestine was playing a role in stimulating the pancreas to secrete enzymes. This action of CCK was shown to be associated with a direct receptor binding phenomenon (Sankaran, Goldfine et al. 1980). They showed that in rat pancreatic acinar cells a high affinity receptor (CCK-A-type) exists. Also, the CCK receptor in this tissue was shown to bind to gastrin in some species (Fourmy, Zahidi et al. 1987).

The signal transduction mechanisms involved in CCK's physiological response were examined in humans by Adler, Beglinger et al. (1991). They showed that atropine or loxiglumide inhibited the response administered prior to exogenous CCK or to meal-induced endogenous CCK stimulation. Similar findings were reported by Schwarzendrube, Niederau et al. (1991). From these results, CCK was proposed to act not only directly, but also via neuromodulation using the cholinergic pathway. There is evidence of species variation regarding a pure hormonal or combined neural/hormonal combination. In dogs, atropine does not alter the response to the exogenous CCK (Beglinger, Grossman et al. 1984) whereas in rats (*in vivo*), endogenously induced CCK pancreatic protein secretion was shown to occur by stimulation of the vagal afferent

pathway (Li and Owyang 1994). This neural component was tested using atropine, acute vagotomy, and chemical ablation of the vagal afferent terminals in rats. The conclusion was that CCK was binding to a CCK-A receptor on the afferent nerve (Moran, Norgren et al. 1990).

There was some suggestion that an indirect inhibitory effect caused by CCK on pancreatic secretion could involve two other peptides: PYY and somatostatin. This was tested by Brodish, Kuvshinoff et al. (1993); and (1995) who showed that PYY and somatostatin inhibited CCK-induced pancreatic exocrine secretion by an intrapancreatic cholinergic mechanism. There are only very few studies completed with these peptides and much more work is needed to prove this theory.

Two models were suggested to explain CCK's direct effect on pancreatic acinar cells, one by Collins, Abdelmoumene et al. (1981) and the other by Sankaran, Goldfine et al. (1982). The model of Collins et al. (1981) depicts two binding sites: a high affinity site, where binding of CCK is mandatory, and a low affinity site, which only binds after the high affinity receptor is occupied. The model of Sankaran et al. (1982) is slightly different, it has a high affinity binding site and a low affinity site, but the high affinity site binds two CCK molecules rather than just one. Recently, Talkad, Patto et al. (1994), added to the model by indicating a third very low affinity site. They report that each site depends on the structural features of various CCK receptors and that each individual site can activate a specific signal-transduction mechanism. The work by Lambert, Svoboda et al. (1985), and Williams, Sankaran et al. (1982) suggested that CCK binding induces an association of the CCK receptor with a guanine nucleotide binding protein that initiates intracellular signal transduction. This was confirmed by the cloning of the CCK-A receptor which was shown to be a G protein-coupled receptor (Wank, Harkins et al. 1992). The CCK-A receptor was located on the basolateral membrane of the pancreatic acinar cells of rats (Williams, Sankaran et al. 1982). CCK action was shown to be mediated through the active subunit

from the G protein combining either to phospholipase C, possibly the β species which is found in three forms (Rhee, Kim et al. 1991), or adenylyl cyclase.

The second messenger IP_3 (1,4,5 trisphosphate form) has also been shown to be involved in acinar cell activation in rats (Rowley, Sato et al. 1990). In addition, intracellular calcium has been linked to acinar cell stimulation, paralleling the effect but not following a simple correlation with IP_3 (Wakui, Osipchuk et al. 1990). This multisignal transduction pathway involved in mediating CCK's effects in this tissue substantiates the conclusions made by Talkad, Patto et al. (1994).

One other noteworthy point, the secretory products of the exocrine pancreatic tissue are primarily digestive enzymes (amylase, proteases, lipase), aqueous alkaline fluid and pancreatic secretory trypsin inhibitors (PSTIs (Kazal-type inhibitor)) which include monitor peptide. Monitor peptide is one of the peptides involved in stimulating the release of CCK from I-type endocrine cells (Bouras, Misukonis et al. 1992). It is likely that CCK's effect on exocrine pancreatic tissue would include an increase in the release of more monitor peptide thus increasing the secretion of CCK. This indicates a positive feedback mechanism for the release of CCK from the small intestine.

E. Effect of endothelins and somatostatin on hexose absorption in the small intestine

This section is included to examine only two of the many possible peptides that may mediate the action of CCK, as was mentioned earlier, (see **Section C** of this chapter), which could then act directly on enterocyte transport.

There are three different endothelin isopeptides ET-1, -2, -3 which are found in a number of species including humans. Synthesis of endothelins occurs in endothelial cells and was identified by the physiological action on vascular smooth muscle, where a slowly developing contraction would occur when purified endothelin was added to the medium

(Hickey, Rubanyi et al. 1985). To date, the known properties of endothelins on the gastrointestinal tract are: biosynthesis, which occurs in the mucosa lining the stomach, myenteric nerves of the ileum, and the myenteric and submucosal plexuses of the colon; ulcerogenicity. Binding studies with endothelins indicate that receptors are located in the blood vessels of the submucosa, mucosa and muscle layers of the human colon, also in the myenteric and submucosa plexi. Actions include vasoconstriction, contraction and relaxation of smooth muscle, stimulation of ion secretion, and the stimulation and potentiation of acetylcholine release and action (Rubanyi and Polokoff 1994). Recently, ET-1 has been shown to decrease hexose absorption within 30 min. in human jejunal segments (Kuhn, Fuchs et al. 1997). The mechanism involved in the decrease was a reduction in the activity of SGLT1 and it was TTX insensitive. The intestinal and pancreatic peptide, somatostatin, has also been suggested to regulate the rate of nutrient uptake in the intestine (Unger, Ipp et al. 1977). It has been shown that CCK-8-induced activation of antral and fundic D cells can cause the secretion of somatostatin (Lloyd, Maxwell et al. 1994). For these reasons both endothelin and somatostatin were included in these studies.

F. Purpose of the research

A postprandial increase in plasma CCK levels has been shown to inhibit gastric emptying in rats and in humans, leading to a decrease in the rate of overall nutrient absorption. This physiological response suggests that CCK plays an indirect role in regulating glucose homeostasis; therefore I proposed the hypothesis that CCK not only decreases the rate at which the intestine receives carbohydrate, but it could also be involved in regulating the rate at which carbohydrates are absorbed by the small intestine. Also, if an acute regulation of hexose absorption action exists this will extend the role which the small intestine has in regulating plasma glucose concentration. After establishing that CCK

changes the rate of absorption, the CCK-influenced hexose pathway was studied in a diabetic model to determine if there are defects in this pathway. These studies on the small intestine were performed to establish that CCK not only indirectly regulates hexose absorption by regulating gastric emptying but specifically controls the rate that hexoses enter the bloodstream from the small intestine. The findings from the following experiments should provide a better understanding of how mammals maintain a stable plasma glucose concentration during and between meals.

G. Aims of the research

The aims of this project are as follows:

1. Accurately monitor changes in the rate hexoses are transported from the lumen to the vascular bed in a jejunal segment of rat small intestine for an approximate 2 h period.
2. Determine if physiological concentrations of vascularly infused CCK change the hexose transepithelial transport rate.
3. Establish CCK substrate specificity on transepithelial transport.
4. Elucidate the loci of the CCK effect on polarized absorptive cells.
5. Identify if CCK-type receptors are located on enterocytes.
6. Determine if CCK is acting through the adenylyl cyclase pathway.
7. Establish a mechanism involved in CCK regulation on the absorptive cells.
8. Investigate if CCK actions are similar in a diabetic model.

CHAPTER II

II. EXPERIMENTAL PROCEDURES

A. Measuring the transcellular transport rate of hexose(s) going from the lumen to the vascular bed in a segment of rat jejunum

Male Sprague-Dawley rats (200 - 350 g) were supplied by Taconic Farms, Germantown, NY. The rats were fed a standard chow diet (Purina PMI Rodent Food) and water *ad libitum*. Before the start of the experiment food was withdrawn for approximately 24 hours to minimize luminal contents during surgery. The study was approved by the Health Sciences Animal Welfare Committee from the Faculty of Medicine. All rats were anaesthetized prior to surgery using sodium pentobarbital given by intraperitoneal injection (60 mg/kg body weight) and placed on a heated (37 °C) surgical table. The techniques and apparatus used in these experiments were modified from those described by Bronk and Ingham (1979) and Pennington, Corpe et al. (1994). After performing a laparotomy, the blood supply to the spleen, rectum, colon, cecum, stomach, and ileum were tied off and the tissues removed; the vasculature to the pancreas and duodenum were also ligated. A 35 cm segment of jejunum starting 5 cm distal from the ligament of Trietz was isolated and the luminal contents removed by gently flushing with 20 ml warm saline (0.9 %), and the jejunum was cannulated at both ends. The lumen was perfused with a Krebs-bicarbonate saline[†] solution using a GILSON Minipuls 2 pump (Mandel Scientific Ltd). The solution contained 5 mM 3-O-MG or 1 mM L-glucose and 5 mM 3-O-MG, or 5 mM D-glucose, or 1 mM L-leucine or 5 mM 3-O-MG and 5 mM D-fructose which was maintained at 37 °C, gassed with 95% O₂, 5% CO₂. Isotopically labelled hexoses (10 µCi of either [³H], or [¹⁴C]-labelled hexose, or both), were added to the luminal circuit immediately after portal vein cannulation.

[†] 120 mM NaCl, 4 mM KCl, 2.5 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 1 mM CaCl₂

The single-pass luminal circuit was perfused at a flow rate of 1.6 ml/min, and the solution was segmented by 95% O₂, 5% CO₂ gas bubbles. The gas bubbles were introduced into the luminal perfusate through a Y piece at a flow rate which ensured that bubbles occupied the diameter of the perfusion tube. This not only exposed the tissue to a saturating gas partial pressure, but also helped to mix the solution in the tissue lumen. After a single-pass through the segment of jejunum the luminal perfusate was discarded. The aorta, proximal to the superior mesenteric artery, was ligated just prior to insertion (within 15 sec) of a cannula into the superior mesenteric artery. The single-pass vascular circuit was perfused at a rate of 1.6 ml/min with fresh Krebs-bicarbonate saline solution containing 5 mM D-glucose, 0.034 mM streptomycin sulfate, 5 mM L-glutamine, 1120 USP units heparin and 10 % w/v Ficoll 70 as a plasma expander, which was maintained at 37 °C and gassed with 95% O₂, 5% CO₂ maintaining the pH at 7.4. The experimental design is shown in **Fig.7**. Once the vascular circuit was established, the rat was euthanized and the vascular perfusate was collected via a cannula placed in the hepatic portal vein[‡]. CCK-8 and other peptides were added to the vascular perfusate, whereas IBMX, 8-Br-cAMP, H-7 and forskolin were added to the luminal perfusate, as described in the appropriate figure legends. The effluent was collected continuously for up to 1.5 h using a GILSON (Mandel Scientific Ltd) fraction collector.

[‡] The sizing of the portal vein cannula was increased only after considerable data was collected; the noted difference was the increased absorptive rates of both SGLT1 specific hexoses and fructose.

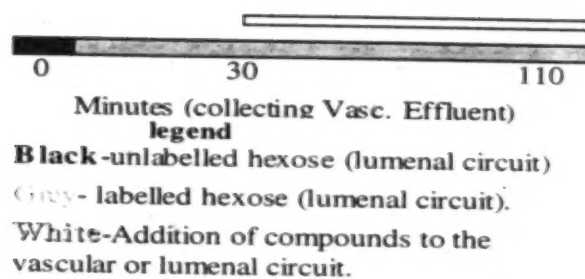


Figure 7. Experimental design of the dually perfused *in situ* preparation.

A diagram of the apparatus and the dual lumenal and vascular circuits used to perfuse a jejunal segment of rat jejunum is shown in **Fig. 8** (modified from Miyazaki, Sunada et al. (1986).

1. *Measurement and calculation of absorption*

The absorption rate for the hexose using the dual perfusion preparation was calculated from the appearance of substrate in the vascular effluent. The experiments were timed from the moment the portal vein was cannulated. Radioactivity of the [^3H] or [^{14}C] samples was determined using a liquid scintillation counter (LS 6500 Beckman). Rates of transport are expressed in μmoles per gram dry weight of tissue per hour and are calculated using **equation 1**.

$$\text{absorption rate} = [(\text{actual (dpm)}/\text{mean standard (dpm)}) \cdot \text{Luminal concentration of substrate (mM)} \cdot (\text{ratio of sample used for standards}) \cdot (\text{luminal flow rate (ml/h)}/\text{dry weight (mg)}) \cdot 1000].$$

Equation 1.

The dry weight of the tissue was measured after drying overnight at 45 °C.

2. *Data analysis and statistical evaluation*

All experiments in which a steady rate of absorption failed to occur (a 25 % decline after 10 min from maximum rate of absorption) were excluded from the study. In most cases the absorptive rate would either hold steady after the maximum rate was established or a slight decrease would be seen ($3 \pm 0.7 \%$) after 6 min followed by a

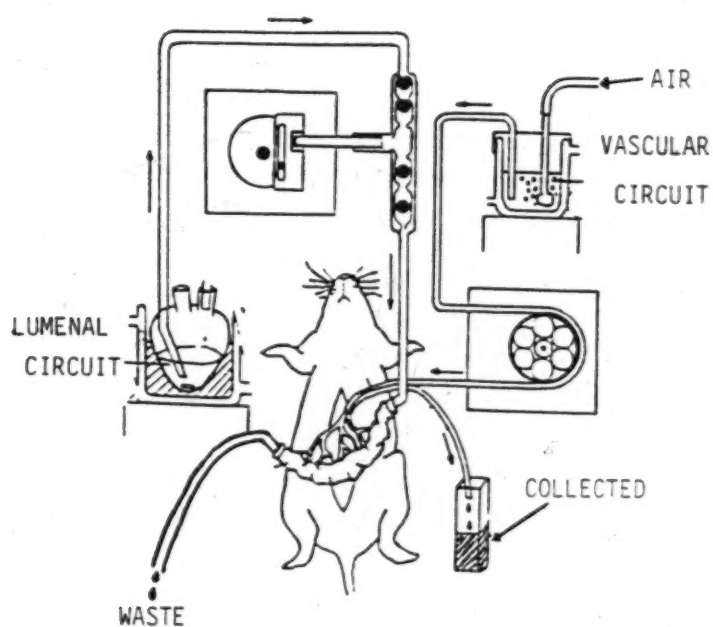


Figure 8. Diagram of apparatus and luminal and vascular circuits used *in situ*.

steady-state rate, which was considered acceptable. All statistics were performed using the absolute value for transport rates and graphically represented as such, except when comparisons are made where the results are expressed as % absorbed (the steady state rate of transport is equal to 100). Means and SEM were calculated from independent experiments and displayed as symbols or vertical bars, as indicated. Statistical analysis was performed by use of repeated measures of analysis of variance. The half-time for decreasing absorption, or half-life ($t_{1/2}$) values were calculated by fitting the rate of decay on a single exponential decay curve (Enzfitter) using **equation 2** (Rang and Dale 1987),

$$t_{1/2} = \ln 2 / Kel$$

where **Kel** is the rate constant, and **ln** is the natural logarithm.

Equation 2.

B. Thin-layer chromatography

To determine whether the D-glucose transported to the vascular bed was intact or had been metabolized we used thin-layer chromatography to determine the % recovery of D-glucose in the vascular effluent. Separation was performed using thin layer chromatography with cellulose and a solvent mixture of ethyl acetate-pyridine-water (40, 20, and 40 % v/v respectively) (Lewis and Smith 1969). The concentration of D-glucose was determined by measuring the isotopically labelled sugar using a liquid scintillation counter (LS 6500 Beckman).

C. Estimations of mucosal $[\text{glucose}]_{\text{int}}$ in lumenally perfused jejunum

To confirm if phloridzin, a specific inhibitor of SGLT1, was blocking the entry of glucose across perfused intact absorptive epithelium, it was added to the lumenal perfusate entering a segment of jejunum. The mucosal content of isotopically labelled hexose was measured in two separate jejunal segments of 15 cm length (a control and a phloridzin treated segment) not at the same time. After anesthetizing the rat the lumen of the jejunum was rinsed, each segment was perfused with a Krebs-bicarbonate saline maintained at 37 °C and containing 5 mM [^{14}C] D-Glucose and [^3H] PEG, to calculate extracellular space. As before the lumen was perfused at a flow rate of 1.6 ml/min, and the solution segmented with 95% O_2 , 5% CO_2 bubbles to maintain tissue viability and facilitate mixing. After 20 min. of perfusion with glucose alone, or glucose and phloridzin, the jejunal loops were rinsed, ligated, and removed. Experiments were performed in random order to minimize possible effects of the continued maintenance under anesthesia. The jejunal loop was opened along the mesenteric border and the mucosal lining scraped-off using a glass slide and both wet and dry weights were determined before the radiolabelled substrates were extracted in 0.05 N nitric acid. Radioactivity of samples was determined using a liquid scintillation counter (LS 6500 BECKMAN).

1. *Data analysis and statistical evaluation*

Means and SEM were calculated from independent experiments and displayed as vertical bars, as indicated. The $[\text{glucose}]_{\text{int}}$ was calculated using **equation 3**. Units are in mM.

Estimated [glucose]_m = [D-glucose actual dpm - ((mean D-glucose standard (dpm)/mean PEG standard (dpm)) • PEG dpm) • ratio of sample used for standards].

Equation 3.

The amount of D-glucose in the mucosal cell is corrected for extracellular solute divided by the tissue water (wet weight - dry weight) and expressed per unit dry weight of tissue.

Statistical analysis was performed using Student's *t*-test.

D. Measurement of the rate at which D-glucose is absorbed in lumenally perfused jejunum *in vivo*

The rationale for utilizing this technique was to establish if the rate of hexose absorption as measured *in vivo*, where the vascular supply is untouched, was similar to that measured with the dual perfusion model. Before the start of the experiment food was withdrawn from the animals for approximately 24 h to minimize luminal contents during surgery. All rats were anaesthetized and maintained under anesthetic for the duration of this procedure. All surgery was carried out on a heated (37°C) surgical table. After performing a laparotomy a 35 cm segment of jejunum starting 5 cm distal to the ligament of Trietz was isolated, cleaned by gently flushing with 20 ml warm saline (0.9%), and cannulated at both ends. Each segment was perfused twice, once as a control, and the second period with either 1 mM 8-Br-cAMP present in the luminal circuit or as a repeated control. The luminal perfusate was a Krebs-bicarbonate saline containing 5 mM D-Glucose (10 µCi [¹⁴C]-labelled) and PEG (10 µCi [³H]-labelled). The PEG was used to correct for fluid movement. The solution was heated to 37°C and gassed with 95% O₂, 5% CO₂ to maintain the pH at 7.4. The perfusion system was a single-pass system employing

a flow rate of 1.6 ml/ min, using a GILSON Minipuls 2 pump (Mandel Scientific LTD). Just prior to entering the intestinal segment the solution was segmented with 95% O₂, 5% CO₂ bubbles to facilitate mixing within the lumen. The gas bubbles were introduced into the luminal perfusate through a Y piece at a flow rate which ensured that bubbles occupied the diameter of the perfusion tube. This not only exposed the tissue to a saturating gas partial pressure but also helped to mix the solution in the tissue lumen. The effluent from the luminal circuit was collected at 5 min intervals for up to 35 min using a GILSON (Mandel scientific Ltd) fraction collector.

1. *Measurement and calculation of absorption*

The absorption rate for the lumenally perfused preparation was measured as the disappearance of substrate from the luminal circuit corrected for fluid movement using **equation 4**. Rates of transport are expressed in μ moles per gram dry weight of tissue per min. Radioactivity of all samples was determined using a liquid scintillation counter (LS 6500 Beckman).

absorption rate = [(((PEG (dpm) • ((PEG mean standard • Lumenal concentration of substrate (mM)/D-glucose mean standard)/PEG mean standard)) - ((D-glucose (dpm)/D-glucose mean standard) • Lumenal concentration of substrate (mM))) • lumenal flow rate (ml/min)/dry weight (mg) • 1000)].

Equation 4.

2. *Data analysis and statistical evaluation*

All animals that did not reach a steady rate of absorption were excluded from the study. All statistics were performed using the absolute value of transport rates and graphically

represented as such, except when comparisons are made where the results are expressed as % absorbed (the steady state rate of transport is equal to 100). Means and SEM were calculated from independent experiments and displayed as symbols or vertical bars, as indicated. Statistical analysis was performed by use of repeated measures of analysis of variance.

E. Isotopically labelled hexose washout studies in the dually perfused jejunum

This procedure is similar to the one used by Boyd and Parsons (1978) except the substrate measured was isotopically labelled D-glucose. This washout model is useful for indirectly indicating the locus of CCK action. When the labelled D-glucose is washed-out using unlabelled equimolar concentration of mannitol in the lumen, the rate of washout into the vascular bed can be described by the sum of two exponential terms. The compartments are the fast releasing (vascular flow rate dependent) and the slow releasing (vascular flow rate independent). The setup presents a two compartmental model: **1)** Q_{01} mucosal epithelium layer and **2)** Q_{02} deeper submucosal (muscle) layer. The model will have two kinetics: a fast, K_1 and a slow, K_2 (Boyd and Parsons 1978). The experimental design is shown below (**Fig. 9**).

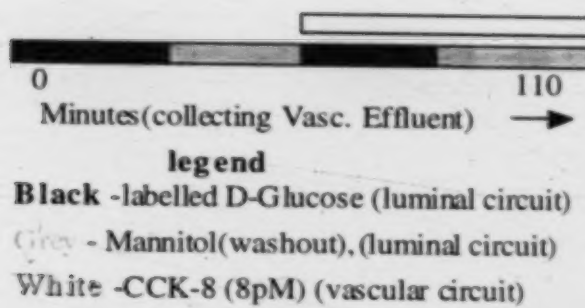


Figure 9. Experimental design for washout studies in the dually perfused *in situ* preparation.

1. *Data analysis and statistical evaluation*

All animals that did not achieve a steady rate of absorption were excluded from the study. All statistics were performed using the absolute value of transport rates. Means and SEM were calculated from independent experiments and displayed as symbols. Statistical analysis was performed by use of repeated measures of analysis of variance and paired *t*-test. Washout data were analyzed using a double exponential decay curve fit (Enzfitter).

F. **Binding study for CCK receptors on isolated basolateral membrane vesicles**

To investigate whether CCK action on hexose absorption is mediated by direct interaction with CCK receptors on the BLM of enterocytes, BLMVs were isolated and ¹²⁵I labelled CCK (Bolton hunter reagent) was used to identify specific receptor binding sites. The segment of jejunum was opened along the mesenteric border and placed flat (villus side up) on a perspex sheet on ice. The mucosa was quickly scraped off with a glass slide and placed in ice cold buffer.

1. *Preparation of basolateral membrane vesicles*

The mucosal scrapings were placed in 60 mls of ice cold MSS (solution A¹). The tissue was homogenized in a Polytron homogenizer (setting 8)(Brinkman) for two minutes. The mixture was poured into centrifuge tubes and centrifuged (Sorval RC5C) at 4,570 rpm for 15 min to remove debris. The lipid layer was removed by suction and the supernatant was centrifuged at 13,100 rpm for 20 min. The supernatant was poured off gently, just until fluffy white pellet is removed. The pellet was resuspend in 3 ml MSS, and homogenized

¹ Solution A: 250mM sucrose, 2mM Tris-HCl, pH 7.4, 0.05 mM PMSF.

for 6 strokes using a hand held homogenizer. 4.2 ml percoll (Pharmacia) was added to the homogenate and topped up to 35 ml with MSS in a centrifuge tube, mixed well, and centrifuge at 20,000 rpm for 1 h. To separate fractions a gradient using a 60% w/v sucrose was used, and collected with a fraction collector (GILSON). The fractions were pooled containing the BLM 4, 5, 6 (as verified with a alkaline phosphatase and a ouabain insensitive Na^+/K^+ ATPase assay) and 20 - 25 mls VRS (solution B^{**}) was added and centrifuged at 20,000 for 30 min and the supernatant was gently suction off. The protein concentration (Biorad) was verified and dilute with VRS to 8.0 mg/ml.

2. *Binding assay for CCK receptors*

The BLMVs were divided into two aliquots, one for specific binding and the other for nonspecific binding. The binding study was performed at room temperature (22°C). BLMVs were placed in microcentrifuge tubes (Fisher Scientific) for a total of 0.5 mg protein. Six fM [¹²⁵I]CCK-8(SO_4) (Amersham, Arlington Heights, IL.) was added along with equal volumes of buffer used for nonspecific binding^{**} and incubated for 3 h. The tubes were then centrifuged at high speed for 1.5 min in the microcentrifuge (Fisher 235A) and the supernatant collected and counted. The pellet was washed using Krebs buffer and centrifuged at high speed, the supernatant discarded and the pellet counted using a gamma counter (Cobra, Camberra Packard).

3. *Data analysis and statistical evaluation*

All data was converted from cps to concentration bound, and plotted to calculate the the bound fraction.

^{**} Solution B: 125mM KSCN, 2mM Tris -HCl, pH 7.4.

^{**} For the nonspecific binding 1 pM - 1μM CCK-8 was included with labelled CCK.

G. Determination of cAMP accumulation in mucosal scrapings

The procedure was similar to the dual perfused procedure as described above. To establish if a link between CCK and cytosolic cAMP concentration exists, mucosal scrapings were analyzed for cAMP content at specific hexose transport rates prior to and after the addition of CCK to the vascular circuit.

1. Tissue preparation

After collecting the vascular effluent to determine the hexose transport rate, the jejunal segment was removed from the animal and opened longitudinally exposing the lumen. The mucosal membrane was rapidly scraped off using a glass slide and placed in preweighed cryovials (Fisher Scientific) and snap frozen in liquid nitrogen. The preparation of tissue extracts was similar to the method used by Steiner, Pagliara et al. (1971). Frozen samples were thawed and homogenized, 6 strokes using a hand held glass homogenizer (on ice), in 700 μ l of 6% trichloroacetic acid (Fisher)(4°C). The samples were then centrifuged at 2500 x g for 15 min and the supernatant fluid removed and extracted 3 times with ethyl ether saturated with water. The aqueous phase was concentrated (speedvac SAVANT) and the residue dissolved in 0.05 M acetate buffer, pH 6.2. The cAMP concentrations were determined using a radioimmunoassay specific for cAMP (Harper and Brooker 1975).

2. RIA for cAMP

For each series of samples a standard curve, 0 -2000 fM cAMP sensitivity, was used to calculate the concentration for each unknown. Using 100 μ l of sample, 10 μ l of a 2:1 triethylamine acetic anhydride solution (Fisher) was added and immediately vortexed. A 100 μ l volume of the first antibody (388 cAMP-specific antibody) was added, vortexed, and then 100 μ l of [^{125} I] cAMP were added and vortexed, and left overnight (4°C). A 50 μ l volume of Sheep Anti-rabbit secondary antibody was added, vortexed and incubated at

4° C for 1 h. Also, 1 ml of PEG was added, vortexed, and then centrifuged at 3200 rpm (RC3C Sorvall) at 4° C for 45 min. The antibody complex solution were then decanted and counted using a gamma counter (Cobra, Camberra Packard).

3. *Data analysis and statistical evaluation*

All animals that did not achieve a steady rate of absorption were excluded from the study. All statistics were performed using the % absorbed and graphically represented as such (where the steady state rate of transport is equal to 100)** for hexose absorption. Means and SEM were calculated from independent experiments and displayed as symbols or vertical bars, as indicated. cAMP concentrations are in pM/mg protein. Statistical analysis was performed by use of repeated measures of analysis of variance and unpaired *t* test.

H. **Measuring SGLT1 protein abundance using Western blotting**

To help identify the mechanism involved in the CCK-induced hexose transport decrease, the abundance of SGLT1 protein in the BBM was measured and correlated with transport rates. Prior to measuring SGLT1 abundance in the BBM the hexose absorption rate was measured using the dually perfused technique and then immediately following the collected fraction the tissue was prepared for determining SGLT1 abundance. The tissue was removed from the animal and the segment of jejunum was flushed with ice cold phosphate buffered saline containing 0.1 mM PMSF and opened along the anti-mesenteric border. The mucosal membrane was rapidly scraped off using a glass slide and placed in preweighed cryovials (Fisher Scientific) and snap frozen in liquid nitrogen.

** The absolute rates of absorption were not calculated as the mucosa was used to calculate the cAMP content.

1. *Preparation of brush-border membrane vesicles*

The frozen mucosal scrapings were thawed at room temperature and then placed in 40 ml of ice cold mannitol Tris buffer (solution A^{††}). The tissue was homogenized (setting 5) in a Polytron homogenizer for two minutes before addition of magnesium chloride to a final concentration of 12 mM. After stirring the solution on ice for 15 min the solution was centrifuged at 4,500 rpm (Sorval RC5C) for 15 min to remove debris. The supernatant was further centrifuged at 16,000 rpm for 30 mins and the pellet homogenized in a mannitol/Tris buffer (solution B^{***}) with a glass homogenizer before further addition of magnesium chloride. After stirring on ice the centrifugation was repeated as before and the pellet was then washed with solution C^{***} before repelleting at 16,000 rpm. This vesicle preparation was diluted in solution C to an appropriate protein concentration, usually 8 mg/ml.

2. *Western blotting*

Membranes (40 µg) from each cell fraction were solubilized in Laemmli sample buffer and run on a 10 % sodium dodecyl sulphate-polyacrylamide gel using a Mini-PROTEAN II cell (Biorad). The proteins were immobilized onto Immobilon-P (PVDF) membrane (Millipore) by electrotransfer, overnight, at 4°C using the Mini Trans-Blot Cell (Biorad). Blocking of the membrane was carried out in 3 % nonfat milk in PBST (solution A) for 1 h and then incubated with 1:1000 rabbit polyclonal antibody to rat SGLT1 (Chemicon International Inc., Temecula, CA) in 3 % nonfat dry milk in solution A overnight at 4°C. The membrane was washed three times in 3 % nonfat dry milk/PBST for 15 min. The Immobilon-P membrane was then incubated with a secondary antibody, anti-rabbit IgG coupled to horseradish peroxidase diluted 1:2,000 in 3 % nonfat dry milk/solution A for 1

^{††} Solution A: 300mM mannitol, 5 mM EGTA, 12mM Tris-HCl, pH 7.4, 0.1 mM PMSF.

^{***} Solution B: 150mM mannitol, 2.5mM EGTA, 6mM Tris -HCl, pH 7.4, 0.05mM PMSF.

^{***} Solution C: 300mM mannitol, 5mM Tris-HCl, pH 7.4

h. Three subsequent washes followed as described above. Finally, the membrane was treated with the ECL detection solution (Amersham Life Sciences) before autoradiography from 1 to 4 mins using Kodak XAR-5 film with an intensifying screen. One distinct band was detected by this method with an apparent molecular weight of 71 KDa.

Solution A: 0.05% Tween 20, phosphate-buffered saline^{***}, pH 7.4

3. *Data analysis and statistical evaluation*

All animals that did not achieve a steady rate of absorption were excluded from the study. All statistics were performed using the % 3-O-MG absorbed and graphically represented as such (where the steady state rate of hexose absorption is equal to 100) for hexose absorption. Means and SEM were calculated from independent experiments and displayed as symbols or vertical bars, as indicated. SGLT1 abundance was determined by quantification of protein density on photographic plate measured using an enhanced laser densitometer (LKB Ultrascan XL₊). The value is arbitrary and was converted to a percentage as compared to control for each individual gel. Statistics were performed using analysis of variance and Newman-Keuls for multiple comparisons.

I. **Chemically-induced diabetes in rats using streptozotocin**

To determine if a link exists between the hyperglycemia which is found in the diabetic animal and the CCK-influenced hexose absorptive pathway, I measured hexose absorption in chemically induced diabetic animals. Sprague-Dawley rats 45 to 50 day old (200 - 250 g)^{***} were anesthetized with Metaphane (methoxyflurane, Janssen Pharmaceutica). After thoroughly cleaning the tail, streptozotocin (Zanosar, Upjohn Don Mills, ON) 65 mg/kg,

^{***} 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 4.3 mM Na₂HPO₄, 1mM PMSF

^{***} The rats were not fasted prior to the administration of streptozotocin.

was administered via tail vein injection. After recovery from the anesthetic rats were fed and watered *ad libitum* for 3 weeks, see experimental design (Fig. 10). To determine if the streptozotocin effectively induced a diabetic state, blood glucose concentrations were measured (Glucoscan, Lifescan Inc., CA) and values above 300 mg/dl were considered as a positive diabetic (80% success rate). All rats used in this series of experiments were used immediately after the 3 week duration.

J. Histological preparation of tissue

Tissue samples were taken from segments of jejunum prior to (control) and after (90 min) luminal and vascular perfusion. Jejunal segments were rinsed in ice cold Krebs buffer and left in 4 % Paraformaldehyde overnight at 4 °C. The samples were then washed in PBS and dehydrated through a graded series of ethanol solutions (100, 70 and 50 %), cleared in xylene and embedded in paraffin wax. For staining, 6 µm sections were deparaffinized in Hemo-De (Fisher) and rehydrated through a series of ethanol solutions (50, 70 and 100 %). Slides were then washed in PBS for 5 min and stained with Hematoxylin (Fisher) and Eosin (Polyscience, Warrinton PA).

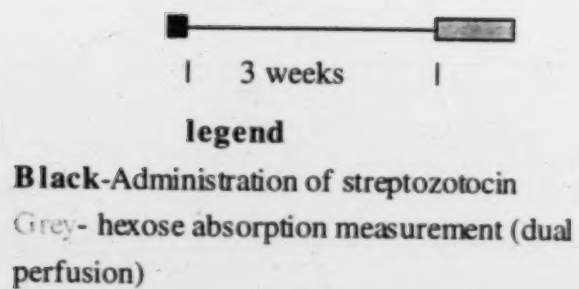


Figure 10 . Experimental design for streptozotocin treated rats.

CHAPTER III

III. RESULTS

To help simplify the organization of this chapter a diagram of the critical experiments performed****, and the areas of research investigated, is shown below (Fig. 11).

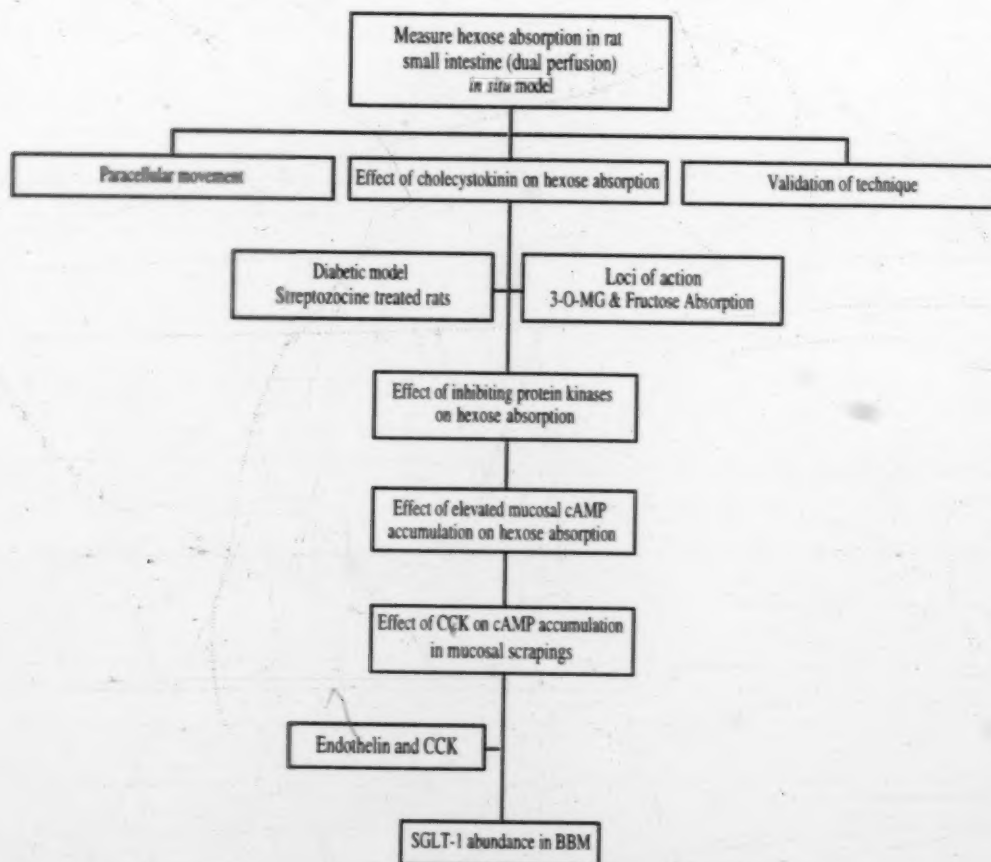


Figure 11. A diagram of experiments performed and areas of research investigated.

**** The diagram is not a flow chart, thus the organization is not in chronological order.

A. Effect of vascularly infused CCK-8 on hexose absorption

1. Validation of the dual vascular and lumenally perfused jejunum

The rationale for using an *in situ* dual perfusion technique was to establish a model that allows an accurate assessment of hexose flux from the lumen to the vascular bed without disrupting other associated tissues, thus maintaining the enterocytes in their normal environment. To assess a change in rate of absorption from the lumen of the intestine to the vascular bed, a defined and reproducible control rate (steady-state level) is essential. The addition of 10 μCi of [^3H]-labelled 3-*O*-MG at the start of the experiment to the luminal perfusate, containing 5 mM 3-*O*-MG, resulted in a rapid appearance of labelled 3-*O*-MG in the vascular effluent (Fig. 12). The rate of appearance reached a plateau within 20 min and then held steady for an additional 70 min for 6 animals (Fig. 12). When isotopically labelled D-glucose was used as the transportable substrate it also appeared in the vascular bed at a steady rate for at least 90 minutes (Fig. 13). The addition of 2 mM phloridzin (a specific inhibitor of the SGLT1) to the luminal perfusate caused a significant decline in 3-*O*-MG absorption from the control rate of $50.0 \pm 0.4 \mu\text{mol/g dry wt/h}$ to $7.0 \pm 0.5 \mu\text{mol/g dry wt/h}$ ($P < 0.001$), with a $t_{1/2} = 13.3 \pm 0.1 \text{ min}$, for 6 animals (Fig. 12). Expressing the rate in the presence of phloridzin as a percentage of the control rate (steady state level) of 3-*O*-MG appearance in the vascular effluent gave a significant decrease from 100 % (steady state rate) to $13.9 \pm 2.0 \%$. The addition of 10 μCi L-glucose to the luminal perfusate containing 1 mM L-glucose had no effect on the rate of transport of 3-*O*-MG and the rate of vascular appearance of this very poor substrate for SGLT1 was much slower, $1.3 \pm 0.1 \mu\text{mol/g dry wt/h}$ for 5 animals (Fig. 12). Also, addition of 2 mM phloridzin to the lumen had no significant effect on L-glucose fluxes across the epithelium: 1.7 ± 0.6 and $1.5 \pm 0.5 \mu\text{mol/g dry wt/h}$, for control, and with phloridzin, respectively (Fig. 12).

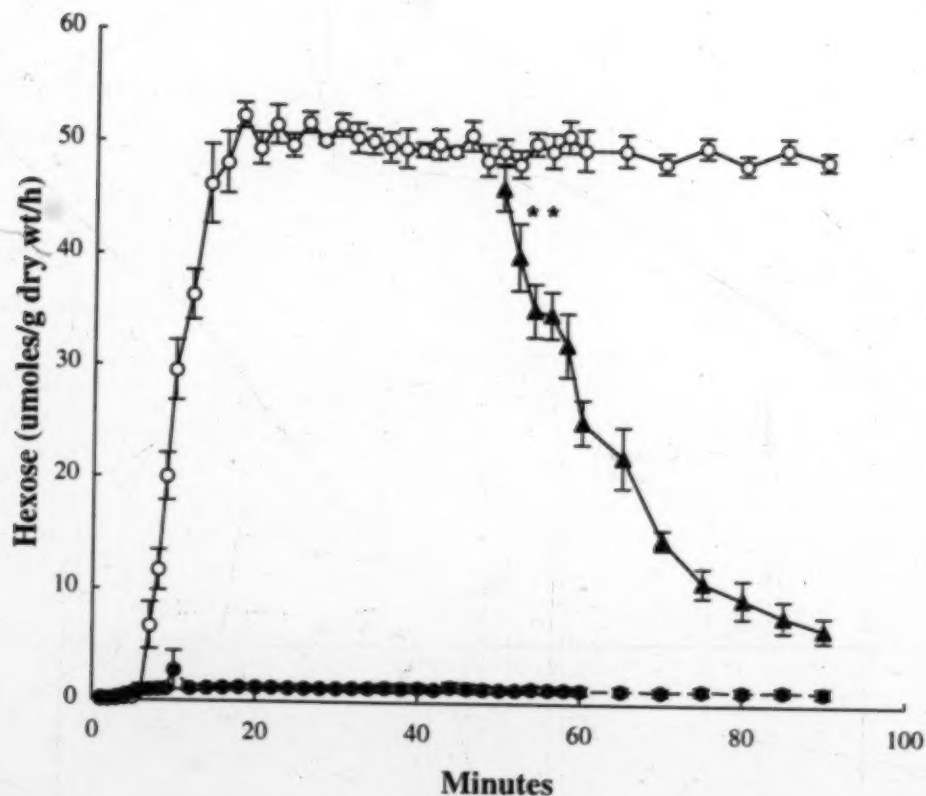


Figure 12. Effect of luminal phloridzin on the steady-state absorption of 3-O-MG and L-glucose in the dually perfused rat jejunum.

The lumen was perfused with Krebs bicarbonate saline, segmented with carbogen gas bubbles, at a flow rate of 1.6 ml/ min. [^3H] labelled 3-O-MG and [^{14}C] labelled L-glucose were added to the luminal circuit (containing 5 mM 3-O-MG and 1 mM L-glucose) within 3.0 min. after cannulation of the portal vein. The vascular bed was perfused with Krebs bicarbonate saline containing 5 mM D-glucose, 5 mM glutamine and 10 % (w/v) Ficoll 70, at the same flow rate as in the lumen (1.6 ml/ min) and the vascular effluent was collected over a 90 min. time period via a cannula in the portal vein. The (○) represents the rate of appearance of the labelled 3-O-MG in the vascular effluent. The (▲) represents appearance of 3-O-MG in the vascular effluent after the addition of 2 mM phloridzin, to the luminal circuit at 55 min. The (●) represents the rate of appearance of the labelled L-glucose in the vascular effluent. Data points represent the mean rate of appearance of 3-O-MG and L-glucose expressed as absorption in $\mu\text{mol/g dry wt/h}$ and the SEM, $n=5$ (** indicates $P < 0.001$, any point following from the same series is significant).

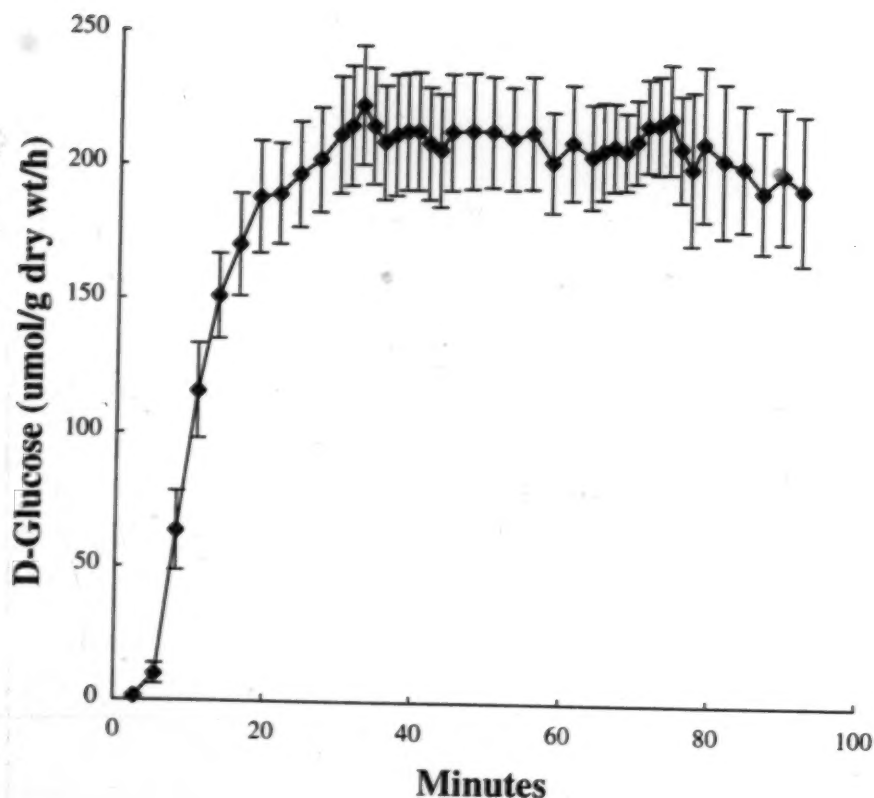


Figure. 13. The steady-state absorption of D-glucose in the dually perfused rat jejunum.

The lumen was perfused with Krebs bicarbonate saline, segmented with carbogen gas bubbles, at a flow rate of 1.6 ml/min. [^3H] labelled D-glucose was added to the luminal circuit (containing 5 mM D-glucose) within 3.0 min. after cannulation of the portal vein. The vascular bed was perfused with Krebs bicarbonate saline containing 5 mM D-glucose, 5 mM glutamine and 10 % (w/v) Ficoll 70, at the same flow rate as in the lumen (1.6 ml/min) and the vascular effluent was collected over a 90 min. time period via a cannula in the portal vein. Data points represent the mean rate of appearance of D-glucose expressed as absorption in $\mu\text{mol/g dry wt/h}$ and the SEM, $n=5$.

To demonstrate that hexoses are transported transcellularly by the transporter proteins, which are believed to be responsible for carbohydrate absorption (Wright, Hirayama et al. 1994), the fluxes of both the SGLT1 specific hexose (3-*O*-MG) and the GLUT5 specific hexose (fructose) were measured simultaneously. Also, the effect of blocking the entry across the SGLT1 transporter was assessed. 3-*O*-MG absorption occurred sooner and was maintained at a greater rate than the absorption of fructose (**Fig. 14**). Adding 2 mM phloridzin to the luminal circuit caused a rapid decline in 3-*O*-MG absorption to a rate 9 fold lower than the steady-state rate; however, fructose absorption showed no decrease, instead a slight increase was observed in the rate of absorption (**Fig. 14**). This experiment was performed in three animals on the absorptive rate of 3-*O*-MG and fructose and similar results were obtained each time^{****}.

^{****} The mean values were not plotted due to other interventions during the experiments.

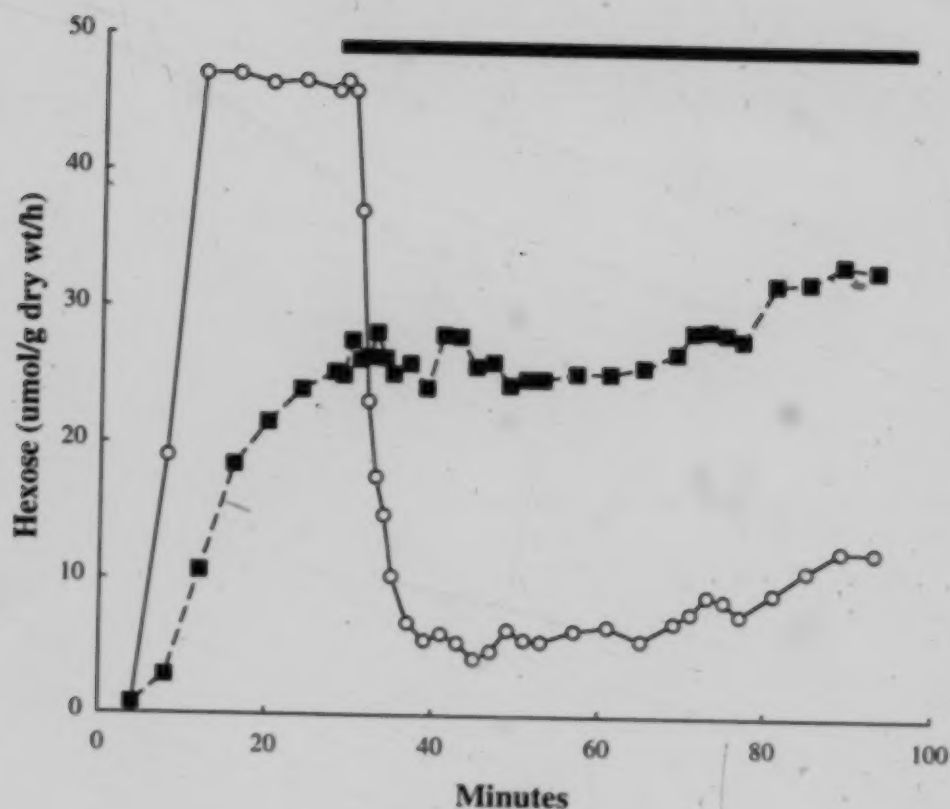


Figure. 14. A representative plot showing the effect of phloridzin on the steady-state absorption of 3-O-MG and fructose in the dually perfused rat jejunum.

The (○) solid line represents the rate of appearance of labelled 3-O-MG in the vascular effluent. The (■) dashed line represents appearance of labelled fructose in the vascular effluent. The horizontal black solid bar represents the addition of 2 mM phloridzin to the luminal circuit. Data points represent the rate of appearance of 3-O-MG and fructose expressed as absorption in $\mu\text{mol/g dry wt/h}$.

To strengthen the reasoning for using a perfusion technique to measure hexose absorption and to confirm the generally accepted model of glucose transport, the cytosolic concentration of glucose was estimated in mucosal scrapings with and without phloridzin added to the luminal circuit. After a 20 min perfusion with 5 mM D-glucose in the presence of 2 mM luminal phloridzin in the lumen there was a 66 % decrease in the estimated concentration of D-glucose in the mucosal scrapings as compared to control values in 3 animals (Fig. 15).

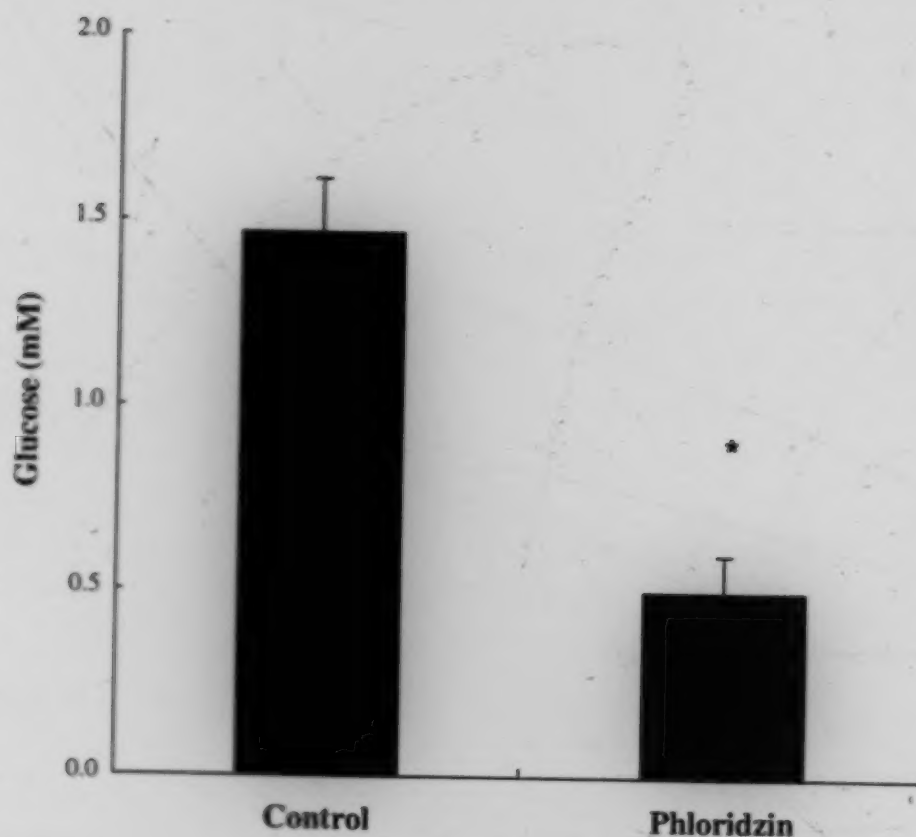


Figure. 15. The effect of phloridzin on the approximate intracellular concentration of D-glucose in the rat jejunum after luminal perfusion with 5mM free D-glucose.

After 20 min. of luminal perfusion with 5 mM D-Glucose and 10 μ Ci [^{14} C]-D-glucose and 10 μ Ci [^{3} H]-PEG the jejunum was excised, rinsed and the mucosa was scraped off. Intracellular glucose was estimated after correction for extracellular substrate. Similarly, intracellular water was estimated from the wet and dry weight of the tissue and corrected for extracellular space. The control bar represents the accumulation of labelled D-glucose in the absence of phloridzin in the perfusate, $n=3$. The 'phloridzin' bar represents the accumulation of D-glucose in the presence of 2 mM phloridzin in the luminal circuit ($n=3$). Results are shown as mean concentration (mM) $\pm$ SEM. * indicates significance $P=0.03$ compared to control.

To establish if the tissue was receiving adequate oxygenation, and thus using cellular respiration as an energy supply for absorbing hexoses, the effects of blocking the oxidative phosphorylation pathway using KCN was examined. Within 25 min after the addition of KCN to the luminal circuit the absorptive rates of 3-*O*-MG^{§§§§} and fructose were decreased to similar and negligible rates (**Fig. 16**). Following an additional ~ 20 min period both sugars were then absorbed at similarly rapid rates where fructose even exceeded its steady-state rate. This procedure was performed twice for 3-*O*-MG absorption and similar results were obtained

One other experiment was performed to determine if the tissue was receiving adequate oxygenation. The Ficoll based Krebs vascular perfusate was replaced with whole blood collected from 5 rats. Similar rates of 3-*O*-MG absorption were recorded and the action of phloridzin was duplicated (data not shown). This indicated that the O₂ carrying capacity of the bubbled Ficoll Krebs solution was adequate for the normal functioning of hexose absorption in this tissue.

^{§§§§} The sizing of the portal vein cannula was increased due to fluid buildup; note the increased absorptive rates of 3-*O*-MG.

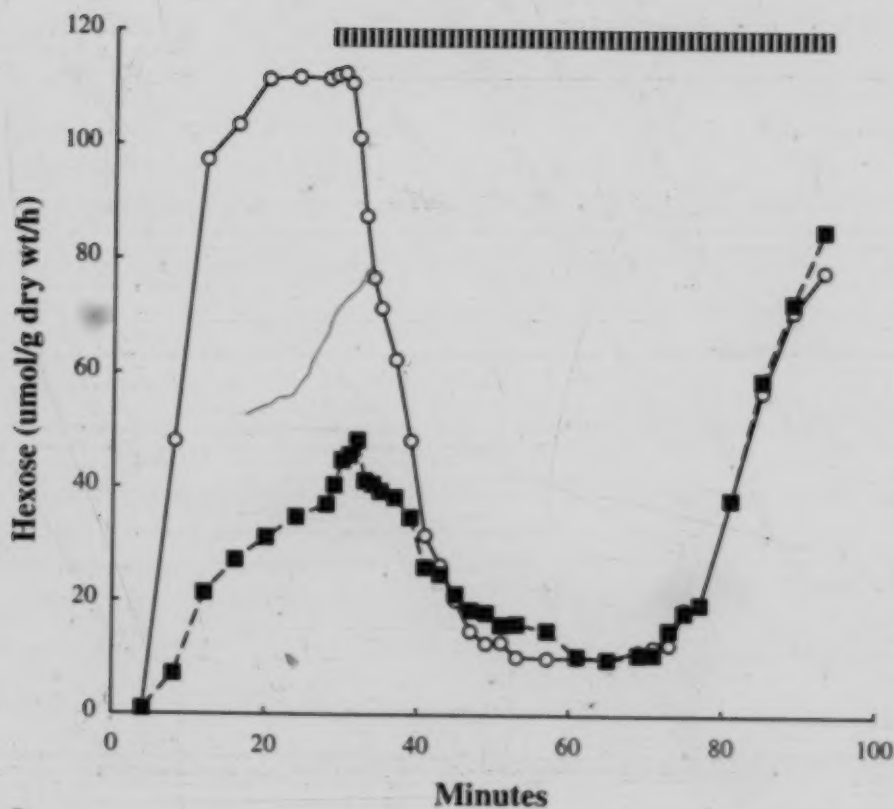


Figure 16. A representative plot showing the effect of blocking oxidative phosphorylation on the steady-state absorption of 3-O-MG and fructose in the dually perfused rat jejunum.

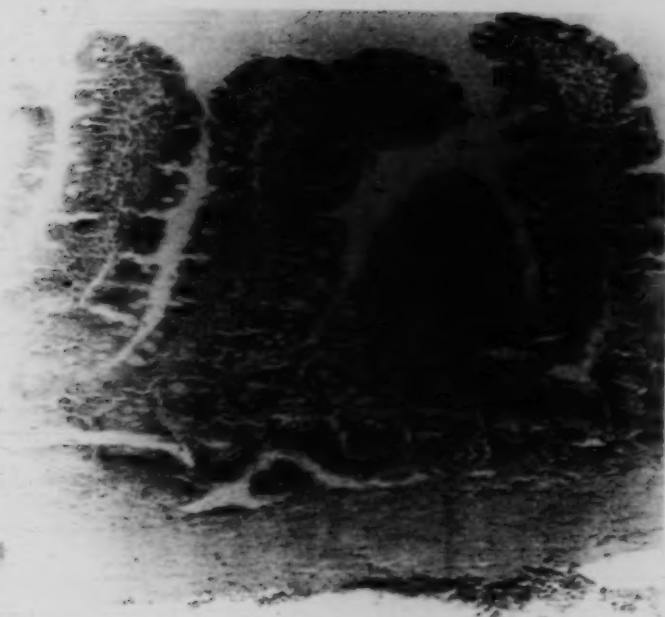
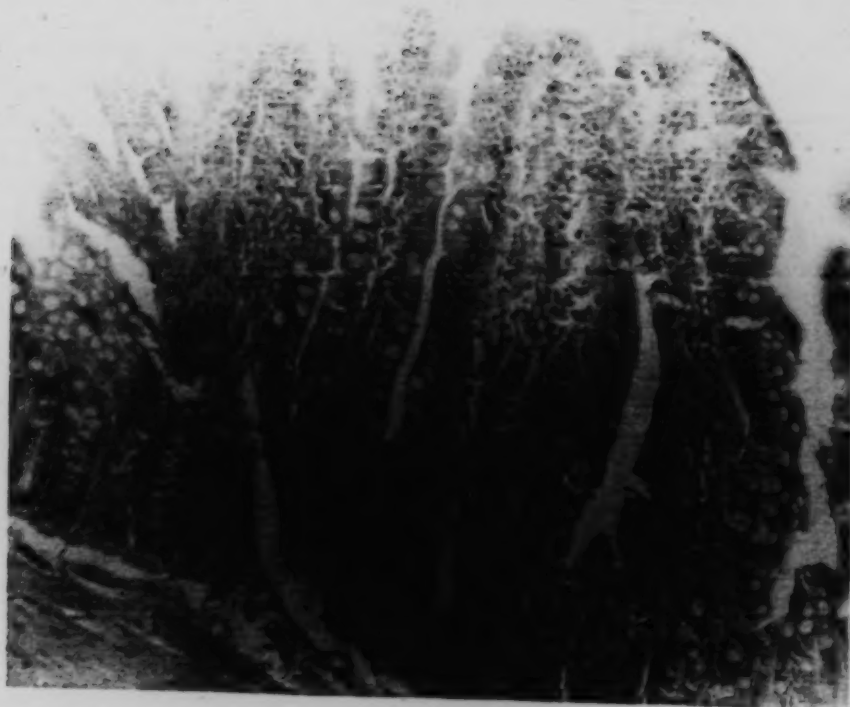
The (O) solid line represents the rate of appearance of the labelled 3-O-MG in the vascular effluent. The (■) dashed line represents appearance of the labelled fructose in the vascular effluent. The horizontal dashed bar represents the luminal addition of 5 mM KCN. Data points represent the rate of appearance of 3-O-MG and fructose expressed as absorption in $\mu\text{mol/g dry wt/h}$.

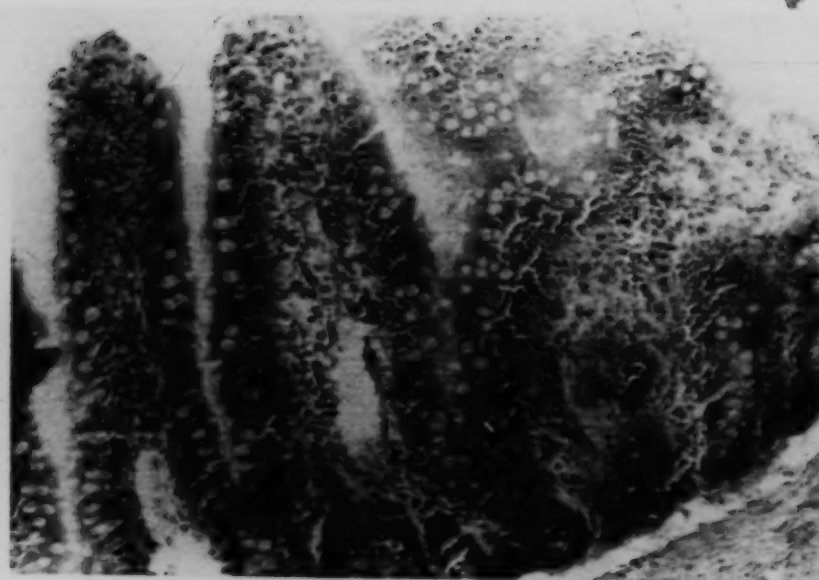
a) Histological comparison of jejunal segments before and after perfusion

To determine if the luminal and vascularly perfused preparation maintained its cellular integrity and arrangement, tissue samples were collected, sectioned, stained, and examined under the light microscope to compare paired segments before and after perfusion. The Figs 17 C,D show that in the perfused animal the villi remain intact and are similar in appearance to the control villi Fig 17 A,B. Also, the simple columnar epithelium remains intact throughout each section as was found in the control sections (Figs. 17 A, B, C, D.).



A

**B****C**



D

Figure 17. A, B, C, and D. Cross sections of rat jejunum before and after dual perfusion.

Tissue segments were mounted in paraffin and cut into 6 μ m sections. The sections were stained using H&E and viewed at a **total magnification x 212**. **A and B** represent the sections taken before perfusion was started (control), **C and D** represent sections taken after 120 min of vascular and luminal perfusion.

At this point I must address the other possible route of absorption across the small intestine, paracellular movement, which is the nonselective movement of various sized molecules through the intercellular spaces via solvent drag. By examining **Fig. 12, 14, and 16** the transport characteristics depicted by using the dually perfused technique provides strong evidence against the paracellular movement pathway. **Fig. 12** shows that L-glucose, which has a similar molecular weight to 3-O-MG, was absorbed at a negligible rate compared to 3-O-MG. Also, when phloridzin was added to the luminal circuit, the absorption of L-glucose was not affected as was also seen in **Fig. 12**, and, there was no evidence of changes in morphology (**Figs. 17 A, B, C, D**). The only indirect indication of paracellular movement was after the tissue was exposed to 5 mM KCN for 50 min (**Fig. 16**). The fructose absorptive rate went from a very low value after 20 min exposure to KCN, equaling that of 3-O-MG, to a rate higher than was achieved prior to the addition of KCN and paralleling that of 3-O-MG absorption.

2. *Effect of CCK-8 on 3-O-MG absorption*

To determine if the small intestine regulates hexose absorption by an enteroendocrine factor such as CCK, CCK at physiological concentrations, was added to the vascular circuit after a steady-state rate of absorption was achieved. Vascularly infused CCK-8 (8 pM) caused a significant decline ($t_{1/2} = 11.3 \pm 0.1$ min.) in the absorption rate of 3-O-MG entering the vascular effluent, ($98.9 \pm 11.6 \mu\text{mol/g dry wt/h}$ at steady state rate to $60.1 \pm 19.3 \mu\text{mol/g dry wt/h}$ ($P < 0.001$) for 5 rats (**Fig. 18**).

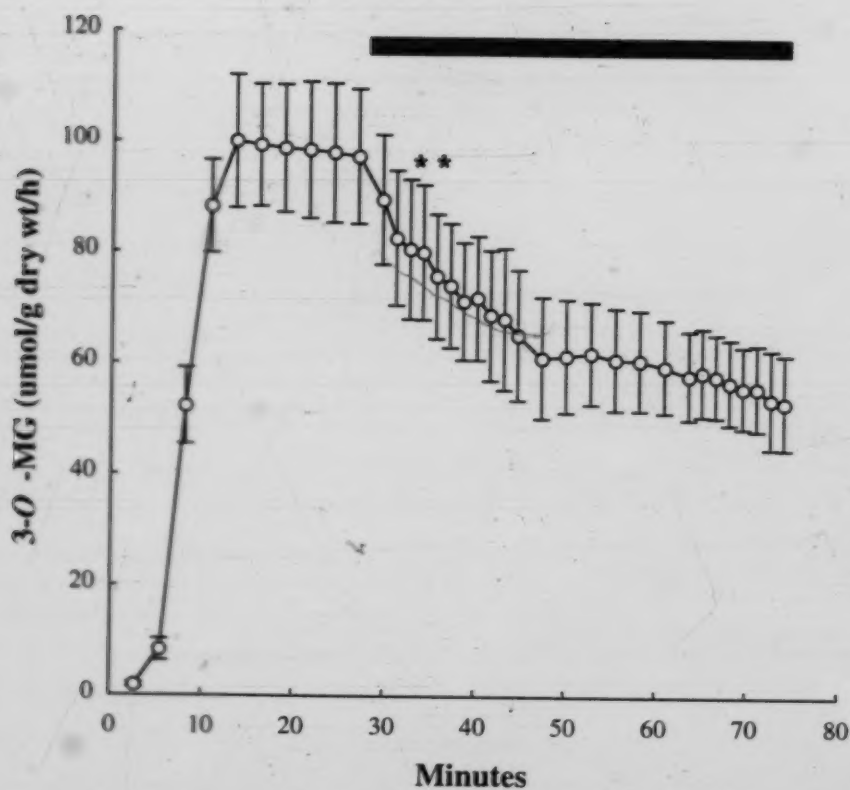


Figure 18. The effect of vascularly infused CCK-8 on 3-O-MG absorption in the dually perfused jejunum.

The (○) solid line represents the rate of appearance of the labelled 3-O-MG in the vascular effluent. The solid black horizontal bar indicates the presence of 8 pM CCK-8 in the vascular circuit. Data points represent the mean rate of appearance of 3-O-MG in $\mu\text{mol/g dry wt/h} \pm \text{SEM}$, $n=5$ (** indicates $P < 0.001$, any point following from the same series is significant).

3. *Dose-response of 3-O-MG absorption to CCK-8*

To establish if the CCK-8 inhibitory influence is dose-dependent we used a range of concentrations of CCK-8 (0.8 to 800 pM) all of which produced significant decreases in the appearance of 3-O-MG in the vascular effluent when compared to the steady state rate obtained in the absence of peptide hormone (**Fig. 19**). CCK-8 at concentrations of 0.8 to 8 pM induced an inhibition of 3-O-MG transport in a dose-dependent manner, ($IC_{50} = 1.8 \pm 0.3$ pM) (**Fig. 19**). The maximum inhibition was seen with 8 pM CCK-8 (**Figs. 18 and 19**) and doses higher than that (80, 800, and 8000 pM) caused a smaller inhibition, 62.7 ± 6.3 % (n=3), 66.8 ± 5.2 % (n=3), and 97 % (n=2) of control, respectively. Because these high concentrations of CCK-8 produced a lower inhibition, indicating a hysteresis, they were not included in the calculation of the IC_{50} .

In contrast, L-glucose absorption was not significantly affected by the presence of CCK-8 in the vascular perfusate, over the concentration range 0.8 - 8 pM, for 3 rats (**Fig. 19**).

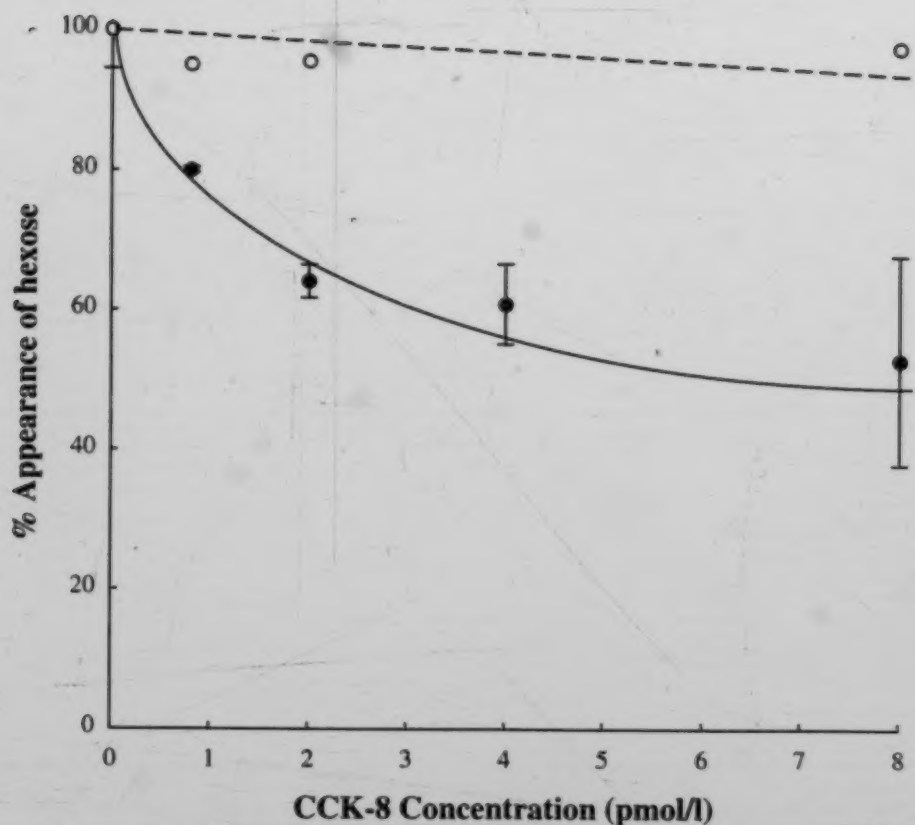


Figure 19. Dose response curve for L-glucose absorption and the inhibition of jejunal 3-O-MG absorption induced by vascular CCK-8 (0.8 - 8 pM). [^3H] labelled 3-O-MG and [^{14}C] labelled L-glucose were added to the luminal circuit within 3.0 min. after portal vein cannulation. CCK-8 (0.8 - 8 pM) was added to the vascular perfusate after approximately 25 min. of perfusion. All data points are expressed as % steady state appearance of control of 3-O-MG and L-glucose (in the absence of CCK-8) in the vascular effluent. Each point (●: 3-O-MG, ○: L-glucose) represents the means $\pm$ SEM $n=3$. The (●) solid line was fitted by nonlinear regression analysis (Enzfitter) assuming a single component and the (○) broken line was fitted by eye. Error bars represent the standard error of the mean and where absent the values are smaller than the symbols.

4. *Influence of CCK-8 on D-Glucose absorption*

To determine if CCK would act in the same way on D-glucose transport, D-glucose was substituted for 3-O-MG in the luminal perfusate. The addition of 8 pM CCK-8 to the vascular circuit caused a rapid and significant decline ($t_{1/2} = 8.0 \pm 0.1$ min.) in the absorption of D-glucose into the vascular effluent (95.6 ± 1.6 $\mu\text{mol/g dry wt/h}$ when compared to the steady-state rate of 210.8 ± 2.1 $\mu\text{mol/g dry wt/h}$) for 5 animals (Fig. 20). Thin-layer chromatography showed that 78 % of radiolabel added to the lumen in the form of D-glucose was unchanged.

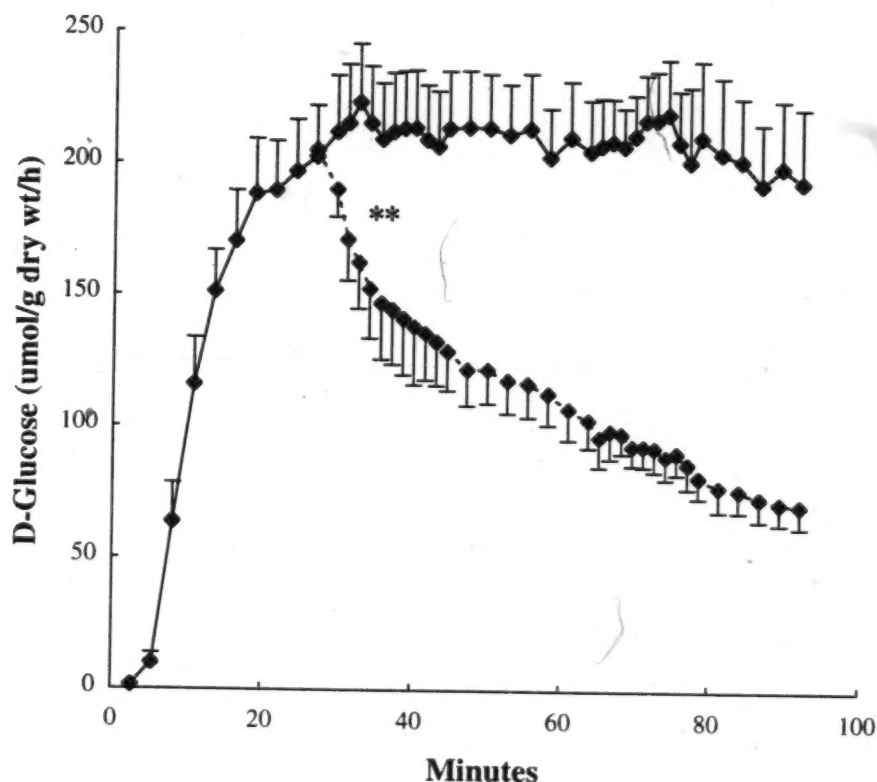


Figure 20. The effect of vascularily infused CCK-8 on the absorption of D-glucose in the dually perfused jejunum. [^3H]-D-glucose (◆) solid line was added to the luminal circuit within 3.0 min. after portal vein cannulation. The dashed line (◊) represents experiments in which CCK-8 (8 pM) was added to the vascular circuit. All data points represent the mean rate of appearance of glucose in the vascular effluent, expressed as $\mu\text{mol/g dry wt/h} \pm \text{SEM}$, $n=5$ (** indicates $P < 0.001$, any point following from the same series is significant).

5. *Effect of CCK-4 on 3-O-MG absorption*

To determine if the CCK-induced inhibition of hexose transport is mediated by A or B-type CCK receptors, two approaches were used: first, CCK-4, a selective CCK B type receptor agonist (Jensen, Qian et al. 1994), was added to the vascular perfusate. Doses ranging from 400 pM to 1600 pM had no significant effect on 3-O-MG appearance in the vascular bed for 5 animal. **Figure 21** shows the very small effect induced by 400 pM CCK-4, and the effect of 1600 pM was no greater.

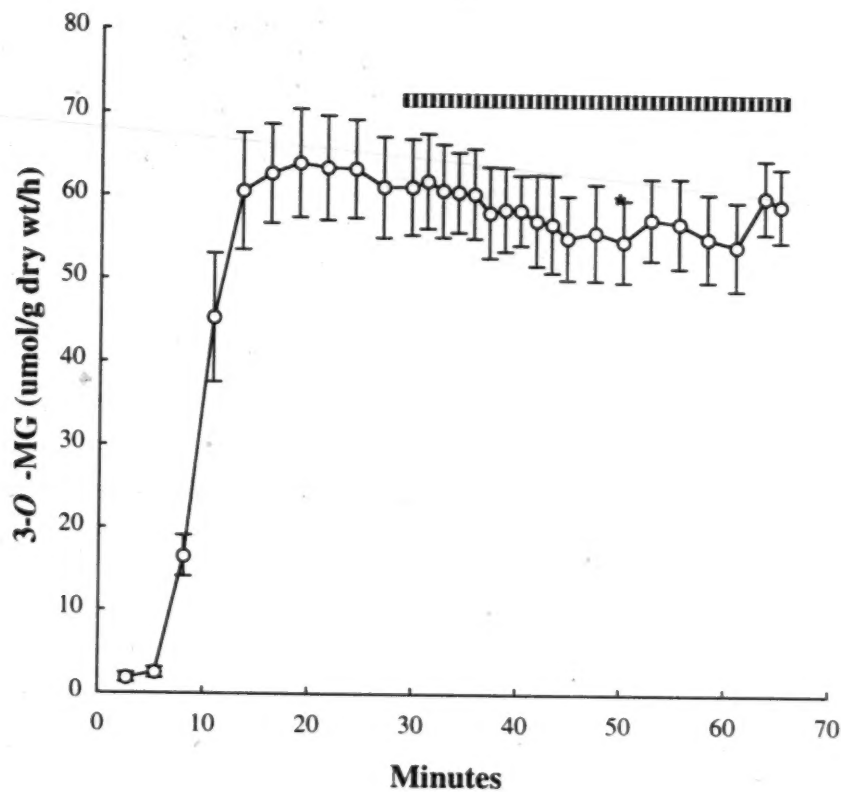


Figure 21. The effect of vascularly infused CCK-4 on 3-O-MG absorption in the dually perfused rat jejunum.

After 30 min 400 pM CCK-4 was added to the vascular perfusate (horizontal dashed bar). All data points represent the mean rate of absorption of 3-O-MG from the lumen to the vascular effluent, expressed as $\mu\text{mol/g dry wt/h} \pm \text{SEM}$, $n=5$ (* indicates a $P < 0.05$).

Secondly, CCK-8 was added to the vascular perfusate after the addition of 2.5 nM CR-1409, a selective CCK-A type receptor antagonist (**Fig. 22**). Previous studies using other tissues have shown that in the presence of 700 pM CCK-8 the IC₅₀ for CR-1409 is 200 nM (Jensen, Qian et al. 1994), therefore 2.5 nM should be a dose sufficient to block a high proportion of CCK-A receptors in this preparation. Comparison of the transport rate in the presence of 1.8 pM CCK-8 with and without CR-1409 showed a significant reduction in the inhibition of the percentage appearance rate in the vascular bed for 5 animals, $p < 0.001$, $93.0 \pm 2.6\%$ and $64.1 \pm 4.1\%$, for 3 animals, respectively (**Fig. 22**). On its own however, the addition of 2.5 nM CR-1409 in the absence of CCK-8 had no effect on the appearance of 3-O-MG in the vascular effluent for 4 animals, (**Fig. 22**).

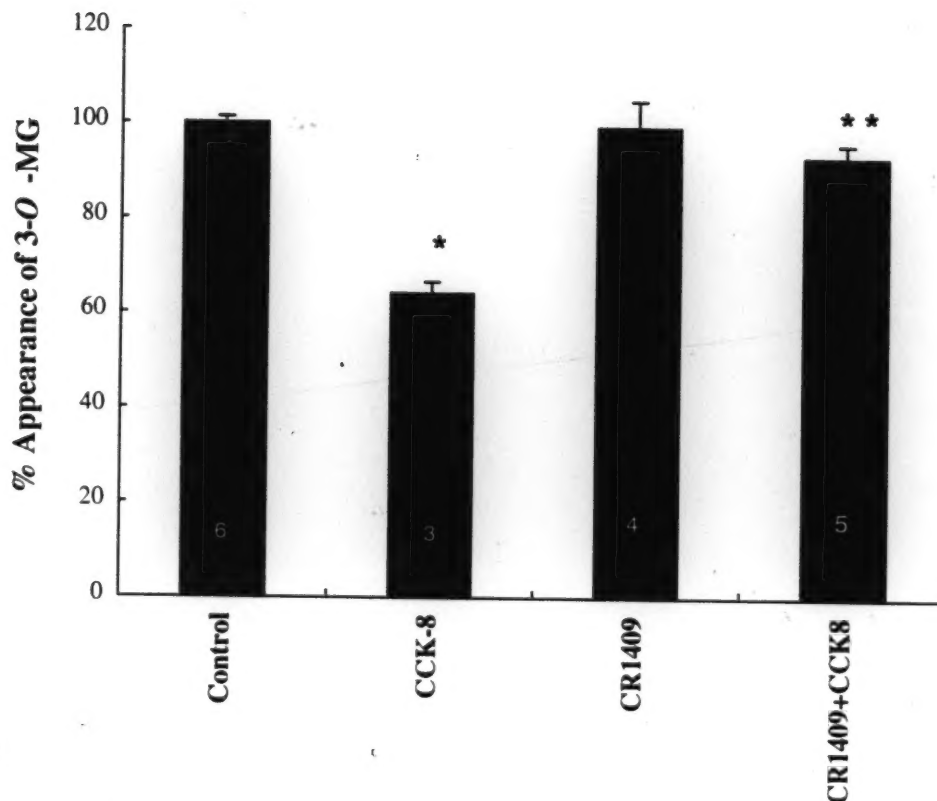


Figure 22. The effect of the CCK-A antagonist (CR-1409) on hexose absorption with and without CCK-8 in the vascular perfusate. Bars represent steady-state rate of appearance of 3-O-MG in vascular effluent draining from dual perfused rat jejunum; the control rate is expressed as 100 %, n=6. CR1409 was added to the vascular circuit at the start of the experiment, CCK-8 was added to the vascular circuit after 30 min. All bars are mean $\pm$ SEM, control bar represents the average value between 20-28 min of perfusion, all others are the average of values between 65-75 min. (* indicates $P < 0.001$, ** indicates a significant difference from the CCK-8 (1.8 pM) bar $P < 0.001$).

6. *Effect of CCK-8 on L-leucine absorption*

To determine if the CCK-induced reduction in hexose absorption is specific for carbohydrate absorption and not a consequence of nonspecific effects such as an alteration of the vascular flow or the Na^+ gradient, the effect of 8 pM CCK-8 on L-leucine (an amino-acid whose transcellular transport is Na^+ -dependent) absorption was also investigated. No significant difference in absorption was observed when 8 pM CCK-8 was added to the vascular circuit, in comparison to control absorption for 5 animals (**Fig. 23**), indicating that CCK-8 has a very selective and specific action regarding nutrient absorption.

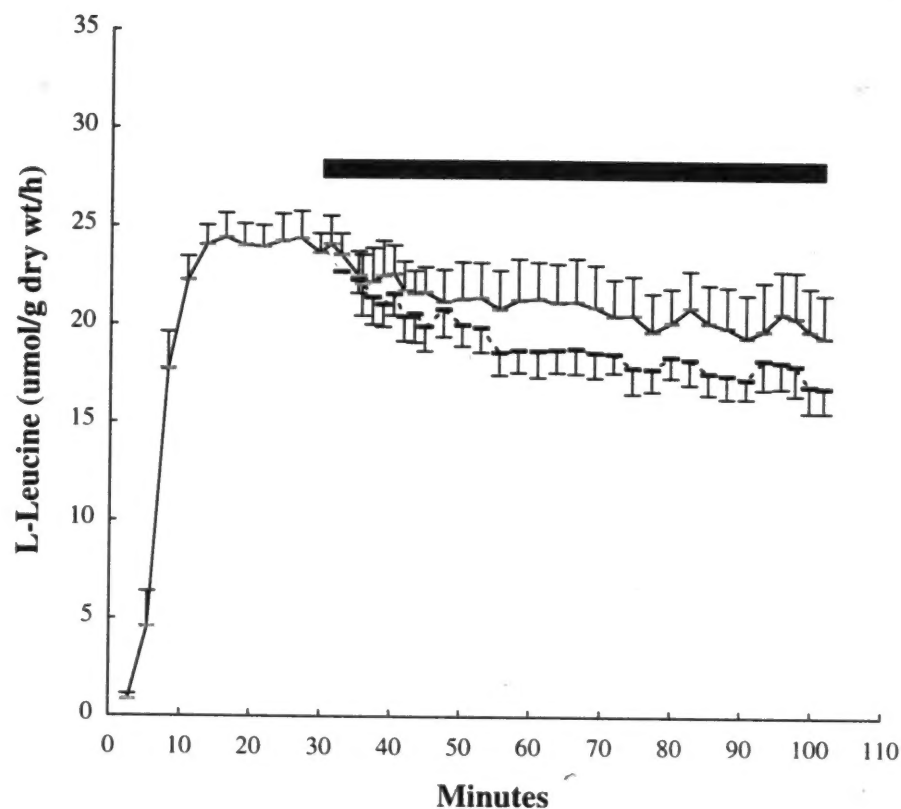


Figure 23. The effect of vascularly infused CCK-8 on L-Leucine absorption in the dually perfused rat jejunum.

[³H]-L-Leucine (—) solid line was added to the luminal circuit within 3.0 min. after portal vein cannulation. The dashed line (---) represents experiments in which 8 pM CCK-8 was included in the vascular circuit (horizontal solid black bar). All data points represent the mean rate of appearance of L-Leucine in the vascular effluent, expressed as $\mu\text{mol/g dry wt/h}$.

7. *Influence of TTX on CCK-8 inhibition of 3-O-MG absorption*

To determine if the CCK response was mediated via a TTX-sensitive intrinsic neural pathway, TTX was added to the vascular perfusate at the start of the experiment. TTX (1 μ M) (Dakka, Cuber et al. 1993) had no significant effect on the steady state rate of 3-O-MG absorption and did not reduce the CCK-8 (8-(80 pM, **Fig. 24**)) inhibition of 3-O-MG transport (67.1 ± 5.1 % of control, $t_{1/2} = 15.1 \pm 1.4$ min.) for 5 animals.

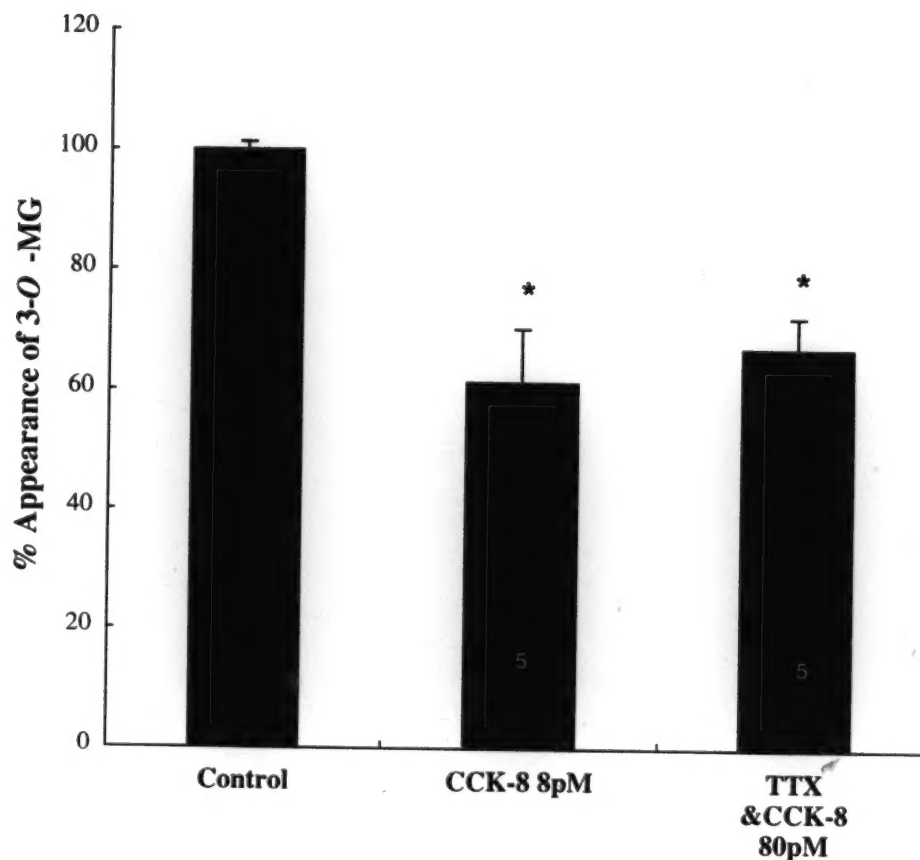


Figure 24. The effect of TTX on CCK-8 inhibition of 3-O-MG absorption. Bars represent the steady-state rate of appearance of 3-O-MG in the vascular effluent draining from dual perfused rat jejunum, where the control rate is 100 % (control value the same as in Fig. 22). TTX was added to the vascular perfusate immediately following portal vein cannulation, CCK-8 was added to the vascular circuit after 30 min. All bars are mean $\pm$ SEM, the control bar represents the average value obtained between 20-28 min, all others are the average of values between 65-75 min. (* indicates $P < 0.001$).

8. *Effect of somatostatin on 3-O-MG absorption*

To determine if the CCK inhibition of hexose transport was mediated through the release of somatostatin, isoforms 14 and 28 were included in the experimental design. Somatostatin-14 (200 - 800 pM) (Berelowitz, Kronheim et al. 1978) had no significant effect on the steady state rate of 3-O-MG appearance (97.6 ± 2.4 %) for 4 animals (**Fig. 25**). However, the tissue was still responsive to CCK-8, because 800 pM in the vascular perfusate caused an inhibition of absorption to 78.7 ± 2.6 % of control. Similarly, somatostatin-28 had no significant effect on the vascular appearance of 3-O-MG (96.6 ± 2.8 % of control) for 4 animals (**Fig. 25**).

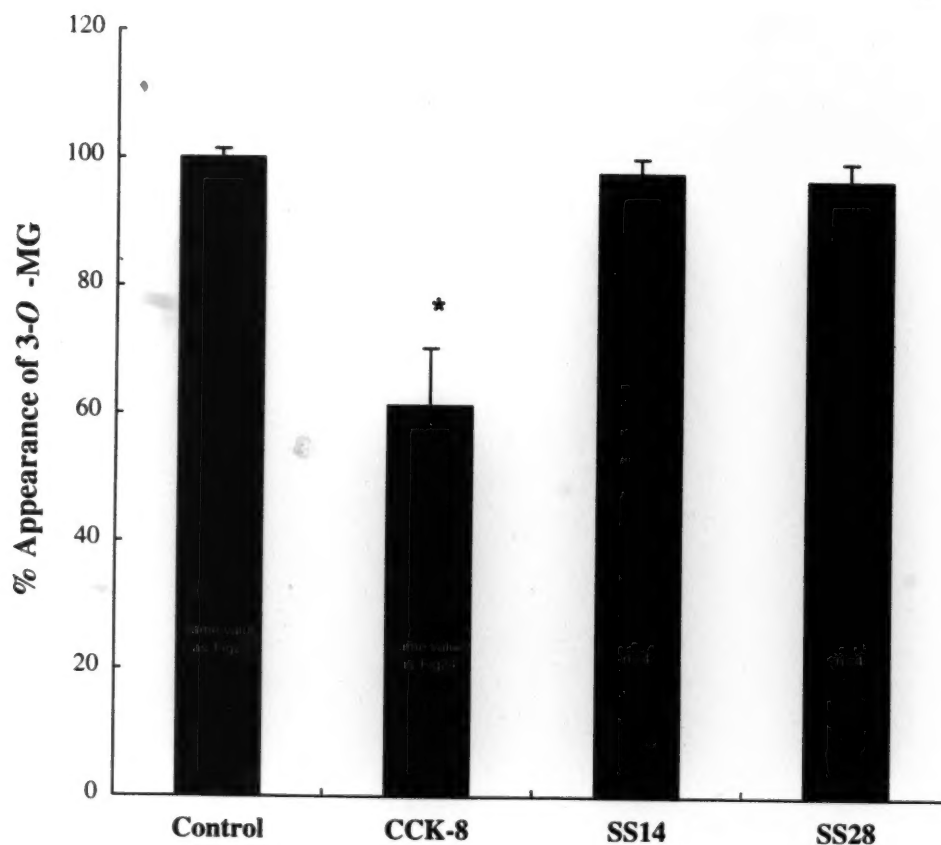


Figure 25. The effect of somatostatin on 3-O-MG absorption in the dually perfused rat jejunum.

Bars represent the steady-state rate of appearance of 3-O-MG in vascular effluent draining from dual perfused rat jejunum, where the control rate is 100 % (control value the same as in Fig. 22). CCK-8 (the same as in Fig. 24), and Somatostatin 14 and 28 were added to the vascular circuit after 30 min of perfusion. All bars are mean $\pm$ SEM, the control bar represents the average of values between 20-28 min, all others are average values from 65-75 min. (* indicates $P < 0.001$).

9. *Effects of CCK-8 on D-glucose washout in the dually perfused jejunum*

To determine the locus of CCK action on the enterocyte the rate of labelled D-glucose washout from the mucosal tissue was analyzed as a two compartmental model. The steady-state rate of D-glucose absorption decreased by 28 % after CCK-8 (8 pM) was added to the vascular circuit (**Fig. 26**). A similar decrease in D-glucose absorption caused by CCK-8 was observed in 3 other experiments.

The luminal washout in the presence of 5 mM mannitol generated a curve which was analyzed using double exponential decay with proportional weighting (Enzfitter). The rate of D-glucose washout for either component (the epithelium or the submucosal muscular layers) was not affected by CCK-8. However, the initial pool size (the vascular flow rate dependant epithelium) was significantly decreased by 37 % (**Table 1**). These results indicate that CCK-8 is acting at the BBM by decreasing the hexose entry.

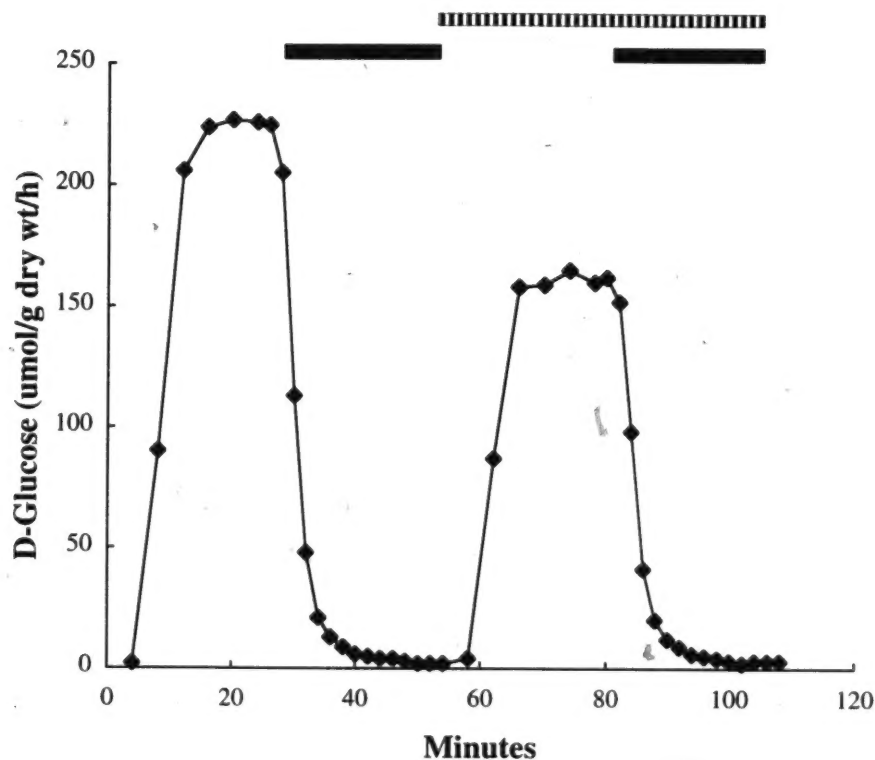


Figure 26. A representative experiment showing the effect of CCK-8 on glucose washout from the rat jejunum.

Within 3.0 min. after portal vein cannulation [^3H]-D-glucose ($\blacklozenge$) was added to the luminal perfusate containing 5 mM D-glucose. The horizontal solid black bar represents the luminal washout in the presence of 5 mM mannitol. The horizontal dashed bar represents the presence of 8 pM CCK-8 in the vascular perfusate. All data points represent the rate of glucose appearance in the vascular effluent, expressed as $\mu\text{mol/g dry wt/h}$.

	Pool size Q_{01}	Rate constant K_1	Pool size Q_{02}	Rate constant K_2
Control (without CCK-8 in vascular circuit)	418±254	0.319±0.041	9±1.3	0.040±0.01
CCK-8 (with 8 pM CCK-8 in vascular circuit)	261 ±148*	0.322±0.044	13±6.9	0.044±0.15

Table 1. Effect of CCK-8 on pool sizes and rates of washout of D-glucose in the dually perfused jejunum.

Control values were obtained from the first washout period, whereas CCK-8 values were from the second washout from the same animal. Values are recorded as mean ± SEM (arbitrary values) and were calculated using double exponential decay (Enzfitter) where the initial values were the pool sizes Q_{01} , Q_{02} , and K_1 was the 1st rate constant and K_2 was the second rate constant (* indicates $P < 0.05$ from control).

10. *Effect of CCK-8 on simultaneous 3-O-MG and Fructose absorption*

To provide further evidence for determining at which pole of the cell the CCK-8 action occurs, fructose absorption was measured simultaneously with that of 3-O-MG. This technique provides direct evidence for which hexose carrier protein is involved in the rate limiting step assuming that: 1. the effects are not occurring on more than one carrier, and 2. the accepted model for hexose transport is valid in this preparation. Fructose did not affect the absorptive characteristics of 3-O-MG (**Fig. 14** and **Fig. 27**). Fructose appeared in the vascular effluent at a much slower rate, $t_{1/2} = 13.7 \pm 0.1$ min, compared to 5.5 ± 0.1 min. for 3-O-MG and fructose maintained a lower concentration, 27.3 ± 3.5 $\mu\text{mol/g dry wt/h}$, than 3-O-MG 75.2 ± 3.9 $\mu\text{mol/g dry wt/h}$ (**Fig. 27**). The addition of CCK-8 (8 pM) to the vascular circuit did not decrease the rate of fructose appearance in the vascular bed, however 3-O-MG absorption was decreased significantly, by 37% (**Fig 27**).

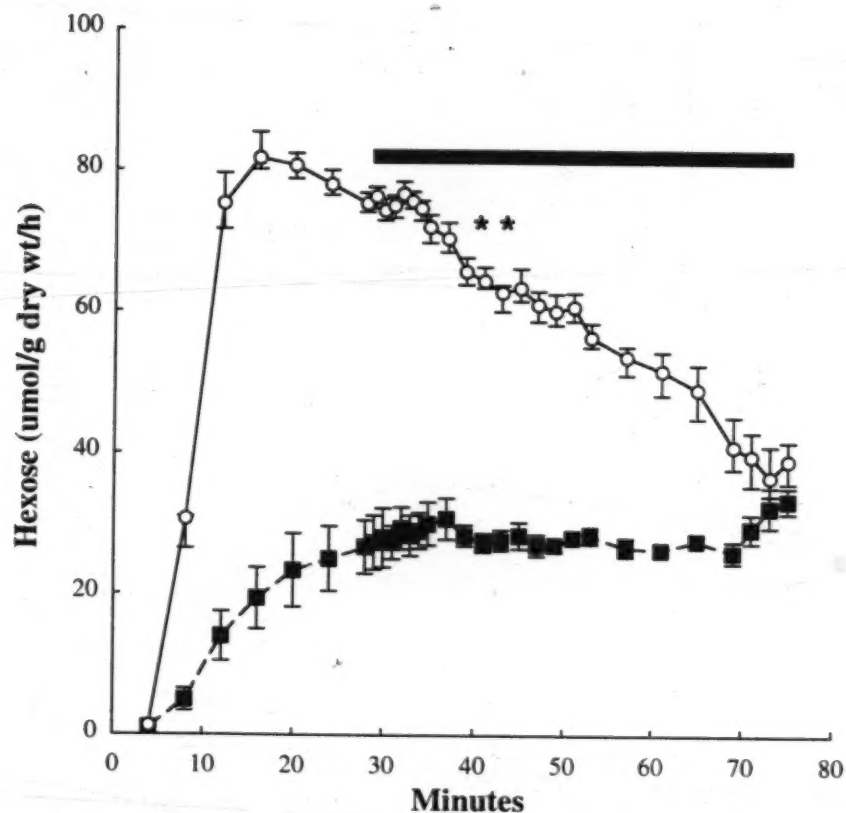


Figure 27. The effect of CCK-8 on simultaneous 3-O-MG and fructose absorptive rates.

The solid line (○) represents the rate of appearance of the labelled 3-O-MG in the vascular effluent. The dashed line (■) represents appearance of the labelled fructose in the vascular effluent. The horizontal solid black bar represents 8 pM CCK-8 included in the vascular perfusate. Data points represent the rate of appearance of 3-O-MG and fructose in the vascular effluent expressed as $\mu\text{mol/g dry wt/h}$ (** indicates significance $P < 0.001$, $n=3$).

11. *Attempts to localize CCK-A type receptors on enterocytes*

To identify if CCK-A high- and/or low-affinity type receptors are located on the enterocyte, the BLM's of the epithelial cells were isolated and binding studies with CCK-8 conjugated to ^{125}I -labelled Bolton-Hunter reagent were performed (**Fig. 28**). ^{125}I -CCK-8 binding to the BLMVs was not competitively inhibited by CCK-8 indicating no specific binding. The half-maximal inhibition could not be calculated from this data because of the erratic scatter of the data.

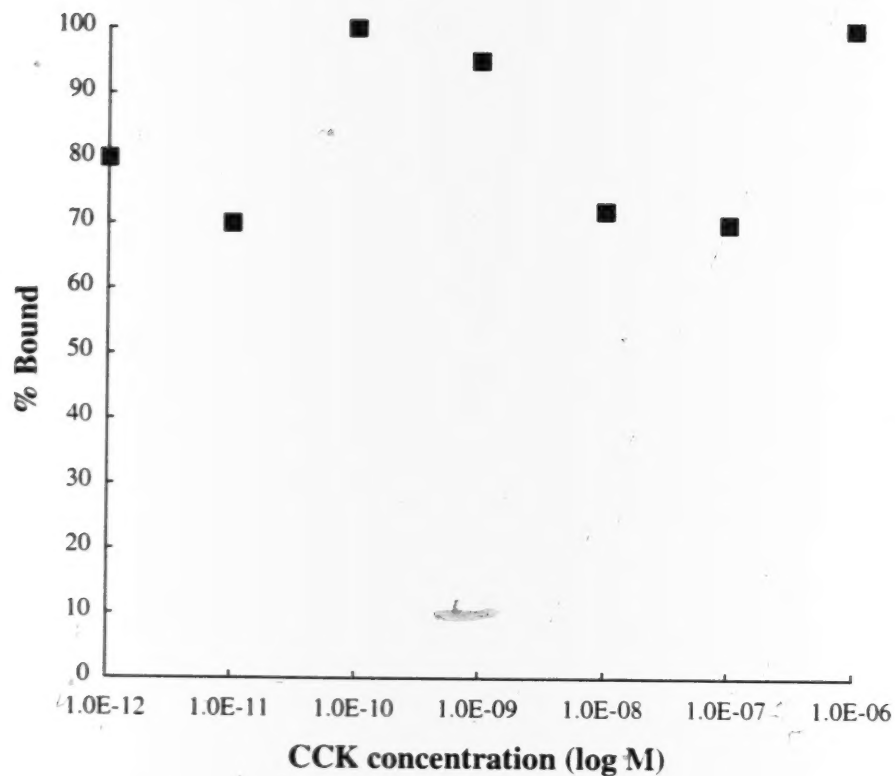


Figure 28 Ability of CCK-8 to inhibit binding of ^{125}I -CCK-8 to BLMVs. BLMV's were incubated with 6 fM ^{125}I -CCK-8 plus the peptide concentration indicated for 180 min at room temperature. 100 % represents the amount of radioactivity saturably bound in the absence of nonisotopically labelled peptide. Values are expressed as a percentage of this amount. Each value represents the mean of triplicate determinations in two separate experiments.

12. *Effect of blocking protein kinases on hexose absorption with and without CCK-8 in the vascular circuit*

To establish if hexose absorption is modulated by protein kinases, and to determine if the action of CCK involves a protein kinase pathway, nonspecific and specific protein kinase inhibitors were included in the luminal perfusate and hexose absorption was measured.

a) The effect of a nonspecific protein kinase inhibitor (H7) on CCK-8-influenced hexose absorption

To determine if a protein kinase is involved in the actions of CCK-8 on hexose absorption, and providing a link between CCK's action and a possible second messenger, a broad spectrum protein kinase inhibitor was added to the the luminal circuit. H-7 (0.1 mM), added on its own, to the luminal perfusate decreased 3-*O*-MG absorption by 30 ± 3 % from the steady-state rate in 3 animals. However, adding 0.1 mM H-7 to the luminal circuit at the start of the experiment abolished the action of CCK-8 on 3-*O*-MG absorption in 4 animals (**Fig. 29**).

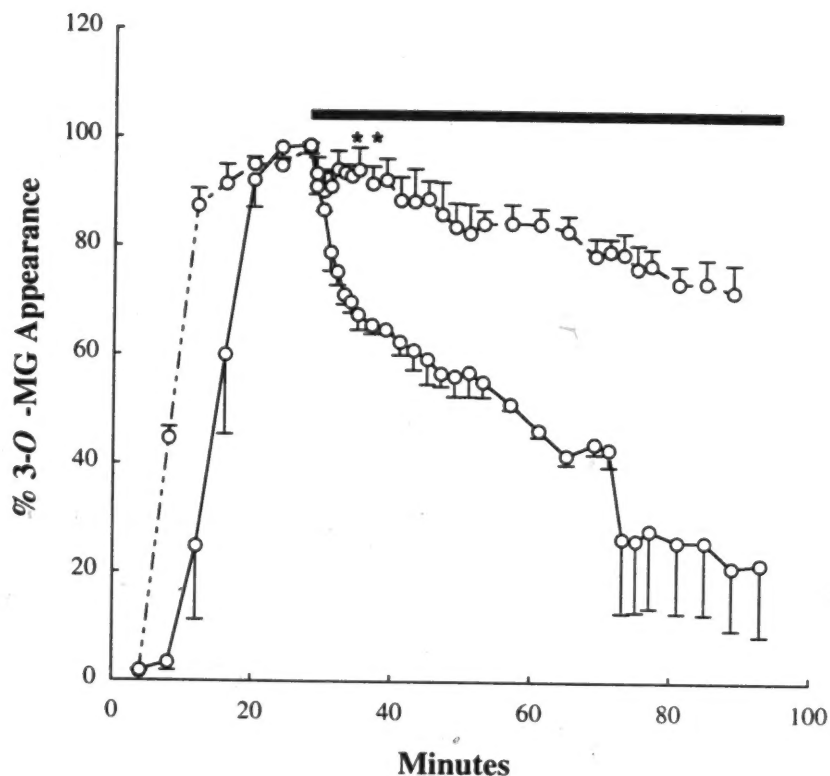


Figure 29. The Effect of H-7 on CCK-8 influenced hexose absorption.

The solid line (○) represents the % appearance of the labelled 3-O-MG before and after the addition of CCK-8 (8 pM) to the vascular effluent (horizontal solid black bar), (n=3). The dashed line (○) represents the % appearance of the labelled 3-O-MG with 0.1 mM H-7 added to the luminal perfusate at the start of the experiment and with CCK-8 (8 pM) added to the vascular effluent (horizontal solid black bar) (n=4). Data points represent the appearance of 3-O-MG expressed as % of steady-state (* indicates significance $P < 0.001$ from the CCK-8 data points, any point following from the same series is significant).

b) The effect of inhibiting protein kinase A on CCK-influenced hexose absorption

To determine if cAMP-dependent protein kinase is involved in CCK-8 action, H-89, a specific protein kinase A inhibitor, was added to the luminal perfusate prior to the addition of CCK-8. H-89 (0.1 μ M) when added to the luminal perfusate caused a significant decrease in 3-*O*-MG absorption (**Fig 30**), but the addition of H-89 to the luminal circuit along with CCK-8 in the vascular perfusate did not show any significant effect on 3-*O*-MG absorption (**Fig. 30**).

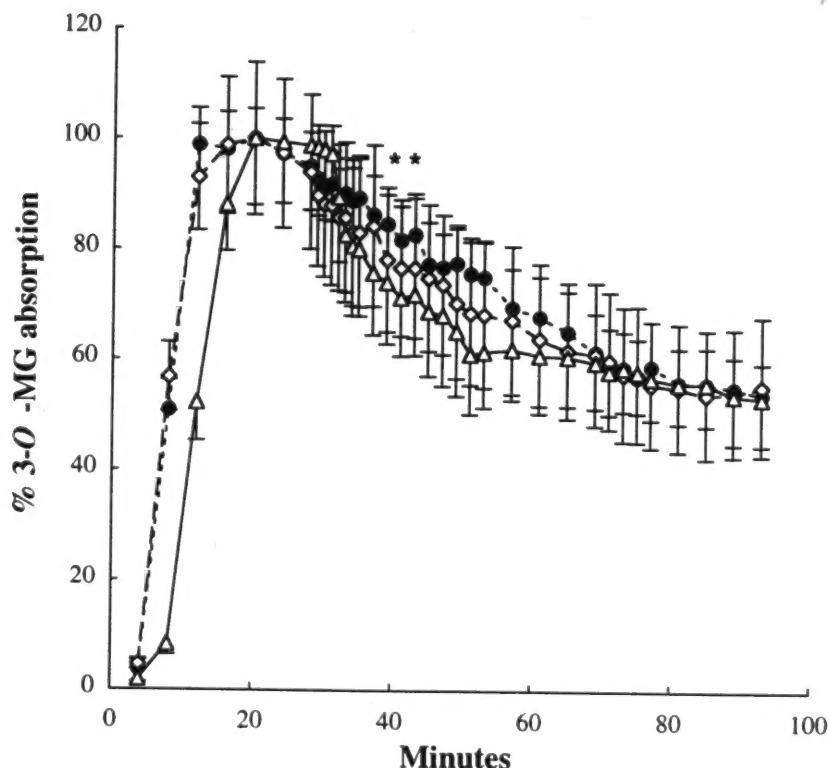


Figure 30. The effect of H-89 on 3-O-MG absorption with and without CCK-8 in the vascular perfusate.

The dashed line (●) represents the % appearance of labelled 3-O-MG when 0.1 μ M H-89 was added to the luminal circuit at the start of the experiment (n=5). The dashed line (◇) represents the % appearance of labelled 3-O-MG with H-89 added to the luminal circuit at the start of the experiment and when CCK-8 (8 pM) was added to the vascular effluent after 30 min, (n=5). The solid line (Δ) represents the % appearance of labelled 3-O-MG with CCK-8 (8 pM) (control response) added to the vascular effluent after 30 min, (n=4). Data points represent the appearance of 3-O-MG expressed as % (* * indicates significance $P < 0.001$ from the steady-state rate, any point following from these series are significant).

c) The effect of inhibiting protein kinase C on hexose absorption

To determine if PKC has an effect on 3-*O*-MG absorption Calphostin-C, a specific PKC inhibitor, was added to the luminal circuit. Calphostin C caused a significant decline in 3-*O*-MG absorption for 5 animals (**Fig. 31**).

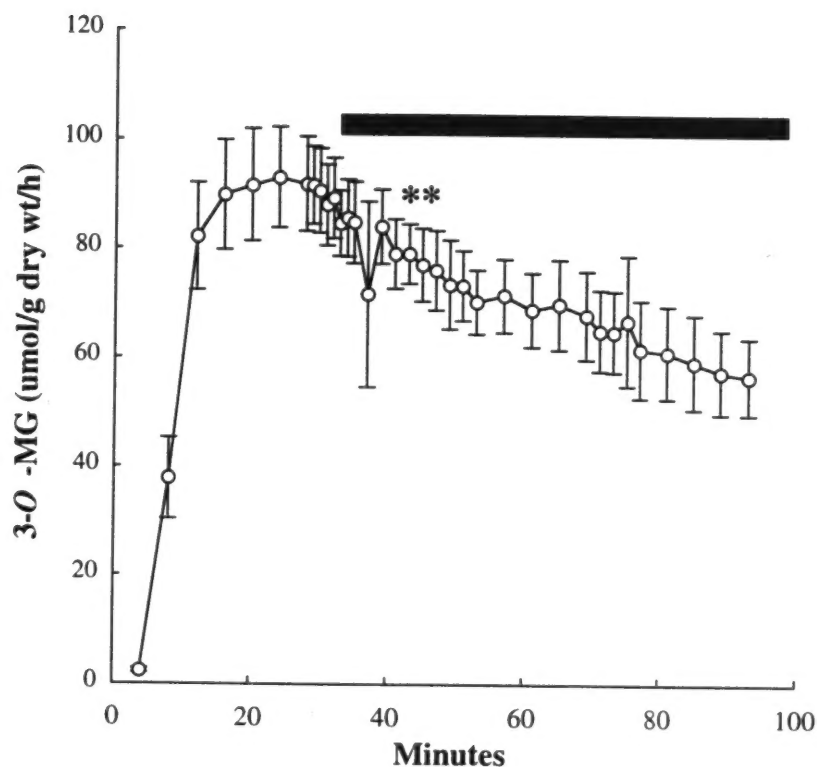


Figure. 31. The effect of Calphostin-C on 3-O-MG absorption in the dually perfused rat jejunum.

After 30 min 0.1 μ M calphostin-C was added to the luminal perfusate (horizontal solid black bar). All data points (○) represent the mean rate of 3-O-MG appearance in the vascular effluent, expressed as μ mol/ g dry wt/ h $\pm$ SEM n=5, (** indicates a P < 0.001, any point following from the same series is significant).

B. Effect of increasing the intracellular cAMP accumulation on hexose absorption

1. Validation of a lumenally perfused in situ preparation

This method is similar to the dual vascular and luminal perfusion technique but has two slight advantages: 1). the animal stays anesthetized for the duration of the experiment, so hypoxic damage to the tissue will be at a minimum; 2). the undisturbed blood supply perfusing the segment of jejunum permits longer experiments and thus allows multiple perfusions of the same segment of jejunum from the same animal. The addition of [^{14}C]-labelled D-glucose to the luminal perfusate containing 5 mM D-glucose at the start of the experiment resulted in a rapid disappearance of the isotopically labelled sugar from the luminal effluent at a rate faster than the appearance of D-glucose in the vascular effluent in the dual perfusion technique (**Fig. 32**). The rate of absorption reached a plateau within 20 min and then held steady for an additional 15 min (**Fig. 32**). There was no significant difference in the absorptive characteristics in a segment of jejunum when perfused for 35 min., rinsed well with Krebs, and then reperfed for the same length of time, for 3 animals (**Fig. 32**).

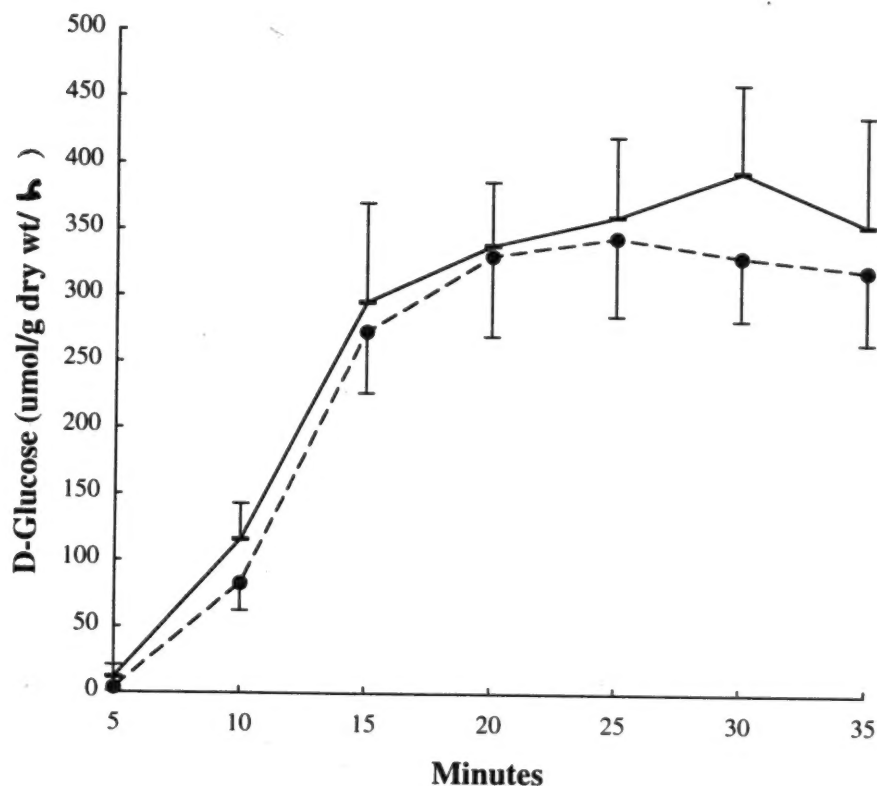


Figure. 32. Absorption^{§§§§} of free D-glucose from the lumen of the jejunum *in vivo*.

[¹⁴C]-labelled D-glucose (—) solid line and [³H]-PEG were perfused through the jejunum containing Kreb's solution with 5 mM D-glucose for 35 min. The luminal effluent was collected every 5 min. The lumen was then rinsed well and the perfusion repeated (● dashed line). Data points represents the mean rate of glucose absorption from the luminal solution corrected for water movement (μmol / g dry wt/ min) ± SEM, n=3.

§§§§ Measured as a rate of disappearance from the luminal perfusate

2. *Effect of 8-Br-cAMP on D-glucose absorption in the lumenally perfused jejunum*

To establish if a rise in mucosal cytosolic cAMP decreases glucose absorption across the small intestine, the cell permeable analogue of cAMP, 8-Br-cAMP (1 mM) was added to the luminal circuit for the duration of the second perfusion. The addition of 1 mM 8-Br-cAMP to the luminal circuit caused a significant $50 \pm 4\%$ decline of D-glucose absorption after 15 min; this was maintained for the duration of the experiment (35 min), for 4 animals.

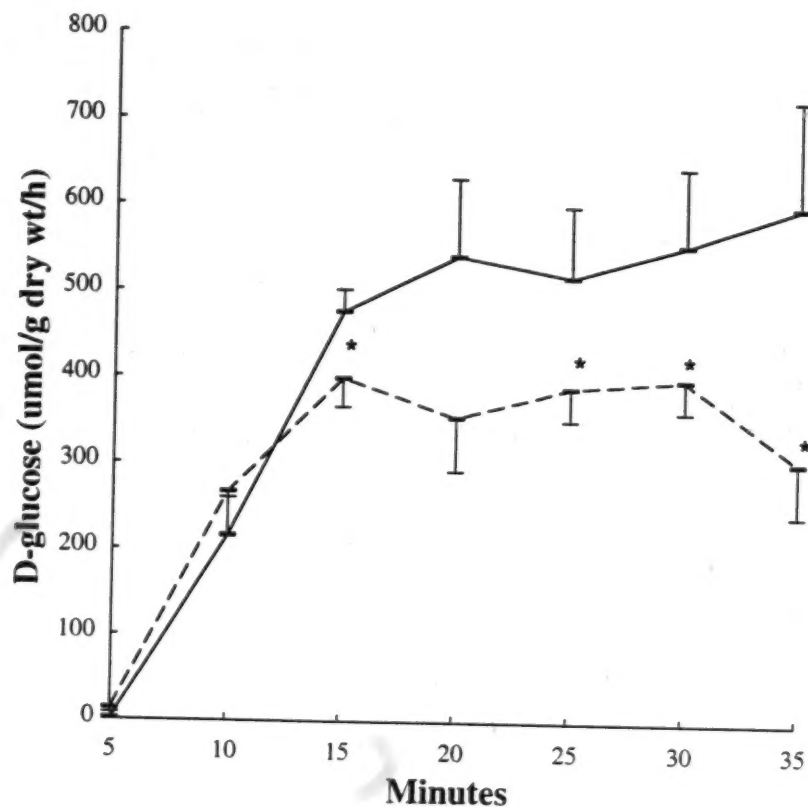


Figure 33. Inhibition of D-glucose absorption by 8-Br-cAMP in lumenally perfused jejunum.

Isotopically labelled compounds were added to the luminal perfusate at the start of the perfusion. Absorption of ^{14}C -D-glucose was measured in the absence (solid line) and presence (dashed line) of 1 mM 8-Br-cAMP in the luminal circuit. Results presented are mean values $\pm$ SEM, $n=4$, * indicates significance $P < 0.05$.

3. *Effect of 8-Br-cAMP on hexose absorption in the dual vascular and lumenally perfused jejunum*

To establish if the effect of 8-Br-cAMP seen in the lumenally perfused preparation is also observed in the dual perfused preparation, and to help identify the site of action of cAMP, fructose and 3-*O*-MG absorption were measured simultaneously (similar to **Figs. 16 and 27**). The addition of the cell-permeable analogue of cAMP (1 mM 8-Br-cAMP) to the luminal perfusate after 30 min caused a significant decline in 3-*O*-MG absorption, but had no effect on fructose absorption.

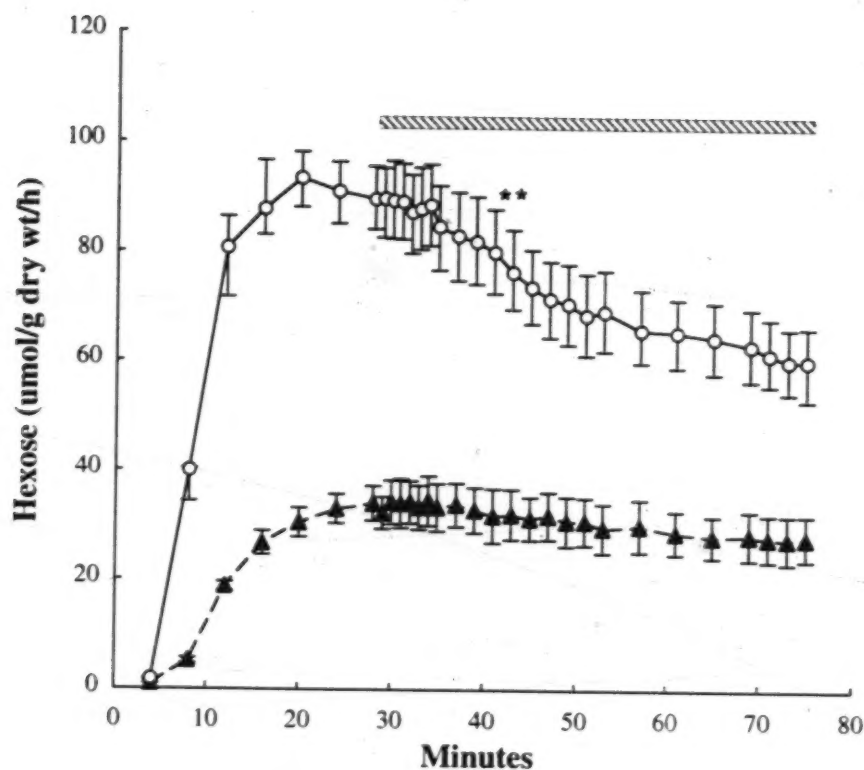


Figure 34. Effect of 8-Br-cAMP on hexose absorption in the dually perfused jejunum.

Isotopically labelled compounds were added to the luminal perfusate after vascular cannulation. 8-Br-cAMP (1mM) as indicated by the hatched horizontal bar was added to the luminal perfusate after 30 min. The absorption rate of 3-O-MG is indicated by the solid line (○), and the rate of fructose absorption is indicated by the dashed line (▲). Results presented are mean values ($\pm$ S.E.M, $n=4$), expressed as $\mu\text{mol/g dry wt/h} \pm \text{SEM } n=5$, (** indicates a $P < 0.001$, any point following in the same series is significant).

4. *Effect of IBMX on 3-O-MG absorption in the dually perfused jejunum*

To provide further evidence that a rise in intracellular cAMP accumulation is associated with a decrease in hexose absorption, IBMX, a non specific phosphodiesterase inhibitor was added to the luminal perfusate. After the addition of 1 mM IBMX to the luminal circuit a significant decline ($28.5 \pm 3 \%$) in 3-O-MG absorption was observed, for 8 animals (Fig. 35).

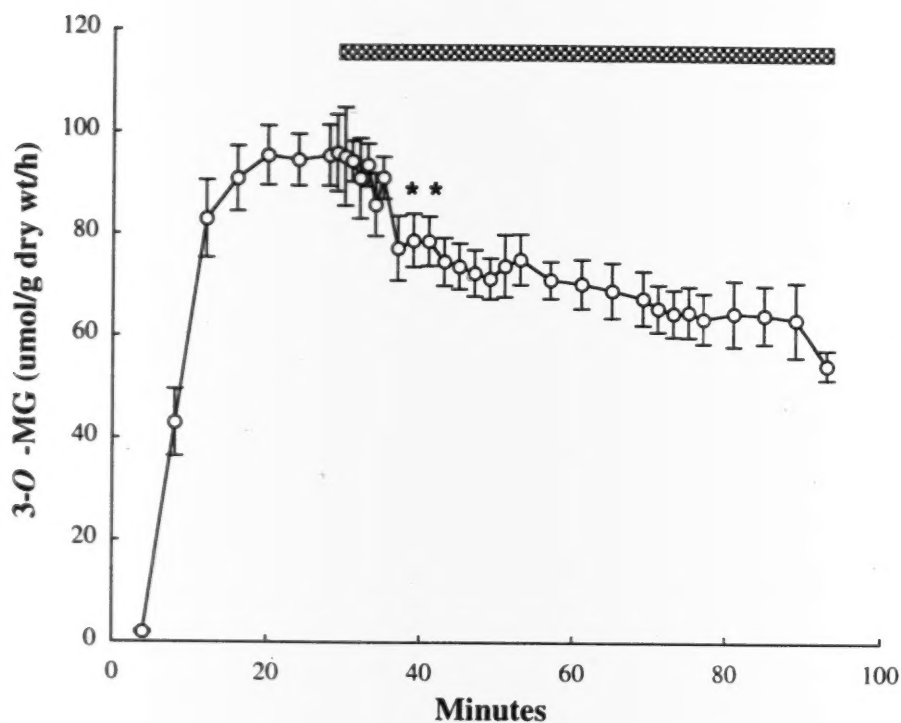


Figure 35. The effect of IBMX on 3-O-MG absorption in the dually perfused rat jejunum.

After 30 min. of luminal perfusion 1 mM IBMX was added to the luminal circuit (horizontal hatched bar). All data points represent the mean rate of absorbed 3-O-MG from the lumen to the vascular effluent, expressed as $\mu\text{mol/g dry wt/h} \pm \text{SEM}$, $n=8$, (* indicates a $P < 0.001$, any points following from the same series is significant).

C. Mucosal cAMP accumulation at various absorption rates

To strengthen the evidence that a rise in cAMP is involved in decreasing hexose absorption in the small intestine direct measurements of both hexose transport and cAMP concentrations were determined on the same tissue sample. The tissue was first perfused then segments were snap-frozen at specific rates(see **Fig. 36** and **37**) of hexose absorption.

1. *The effect of IBMX on 3-O-MG absorption and cAMP accumulation in the jejunum*

At a steady-state rate of 3-O-MG absorption considered as 100 %, the cAMP levels in the mucosa was 362 ± 21 pg/ mg protein, for 4 animals, but after 20 min of 1 mM IBMX luminal perfusion the absorption of 3-O-MG decreased by 26 ± 3.3 %, and the mucosal cAMP concentration increase significantly 4 fold for 4 animals (**Fig. 36**).

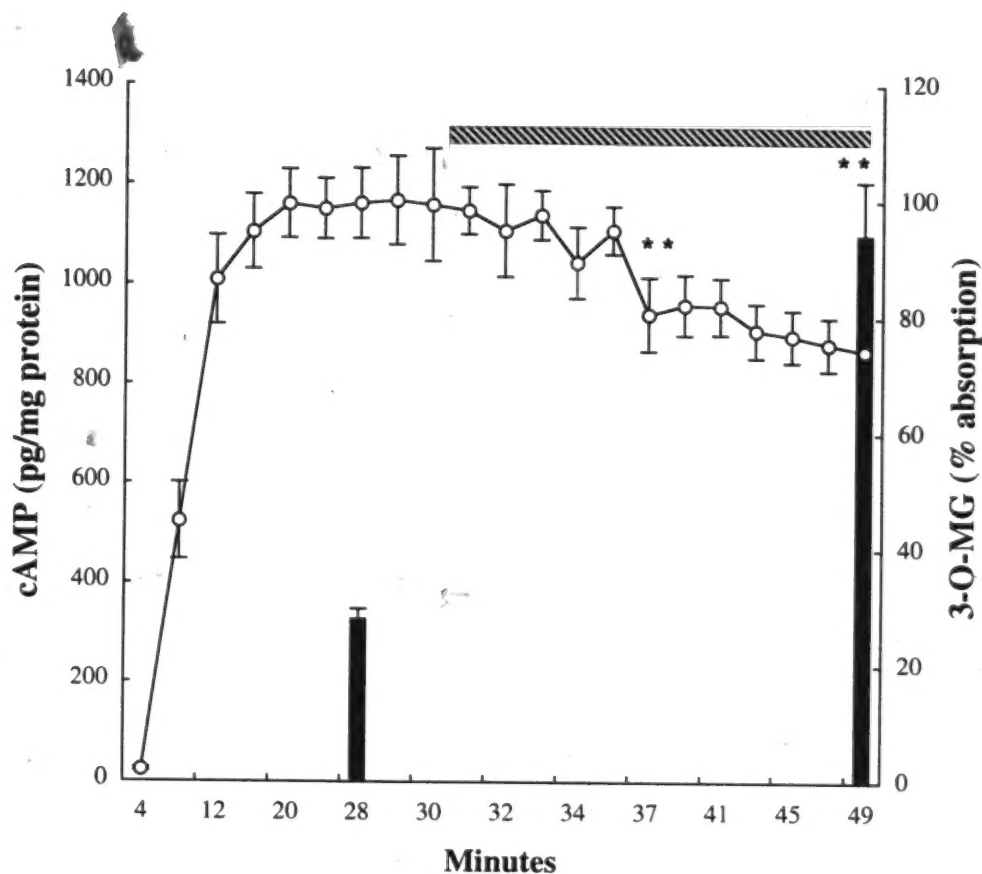


Figure 36. The effect of IBMX on 3-O-MG absorption and mucosal cAMP concentrations in the dually perfused jejunum.

Absorption of labelled 3-O-MG was measured (○ solid line) for the duration of the experiment. 1 mM IBMX was added to the luminal perfusate after 30 min of perfusion (horizontal dashed bar). After 20 min with IBMX present in the luminal circuit the tissue was removed from the animal, rinsed with Krebs solution, opened longitudinally, and the mucosal layer scraped off using a glass slide and cAMP content measured. The steady-state rate bar for cAMP at 28 min. was the mean values $\pm$ SEM, $n=4$ measured prior to these experiments^{*****}. ** indicates significance $P < 0.001$. Results presented are mean values $\pm$ SEM, $n=4$.

^{*****} This data was included to help visualize the changes in cAMP content.

2. *Effect of forskolin on 3-O-MG and cAMP accumulation in the jejunum*

One other experiment performed to determine if an increase in cAMP will decrease hexose absorption was to stimulate adenylyl cyclase directly using forskolin. Forskolin at 0.1 μ M in the luminal circuit caused a significant decrease in 3-O-MG absorption ($19 \pm 3\%$) and significantly increased the mucosal cAMP content by 1.7 fold for 4 animals (Fig. 37).

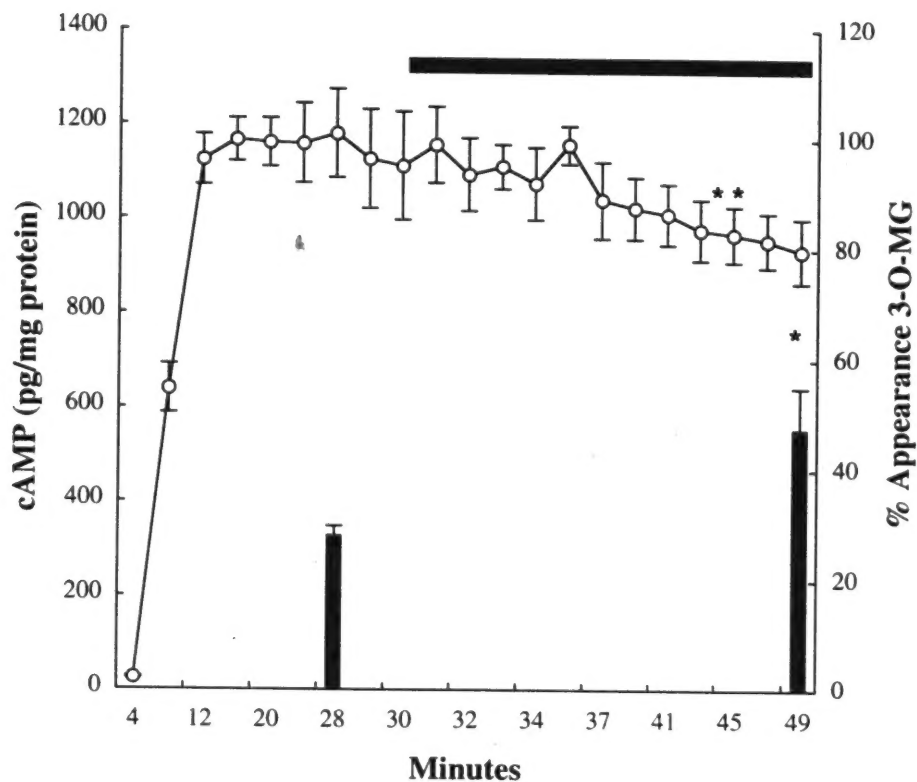


Figure 37. The effect of forskolin on 3-O-MG absorption and cAMP accumulation in the dually perfused jejunum.

Absorption of labelled 3-O-MG was measured (○ solid line) for the duration of the experiment. Forskolin (0.1 μ M) was added to the luminal circuit after 30 min of perfusion (horizontal solid black bar). After 20 min with forskolin present in the luminal circuit, the tissue was removed from the animal, rinsed with Krebs solution, opened longitudinally, and the mucosal layer scraped off using a glass slide and the cAMP content measured. The black vertical bar at 28 min. (mean values $\pm$ SEM, $n=4$) is the same value as in Fig 36).

3. *Effect of CCK-8 on cAMP accumulation in the jejunum*

To provide direct evidence that a rise in cAMP accumulation, caused by CCK-8, parallels a decrease in hexose absorption, both the accumulation of cAMP and hexose absorption were measured simultaneously in segments of jejunum. The addition of 8 pM CCK-8 to the vascular perfusate caused a significant decrease in hexose absorption, for 4 animals (**Table 2.**), but did not affect the mucosal cAMP content (**Fig. 38**). Also, IBMX did not potentiate the accumulation of cAMP with CCK-8 present (**Fig. 38** compared to **Fig. 36** (bar at 49 min.)), but did decrease 3-*O*-MG absorption and cause a significant increase in cAMP accumulation when added to the luminal perfusate (**Fig. 36**).

	Steady-state (control)	CCK-8 (8 pM)	IBMX & CCK-8
3-O-MG absorption (%) (n=4)	100 $\pm$ 2	*68 $\pm$ 4	*65 $\pm$ 3

Table 2. Effect of CCK-8 and IBMX on 3-O-MG absorption in the dually perfused jejunum.

The tissue was vascularly and lumenally perfused and 3-O-MG absorption was measured. Values are the mean $\pm$ SEM, n=4. The tissue was then snap-frozen at a specific transport rate (same as Fig 36) after 20 min of perfusion with either 8 pM CCK-8 in the vascular circuit or both CCK-8 in the vascular perfusate and 1 mM IBMX in the luminal circuit. The mucosal cAMP content was then measured. * indicates significance $P < 0.001$ from control.

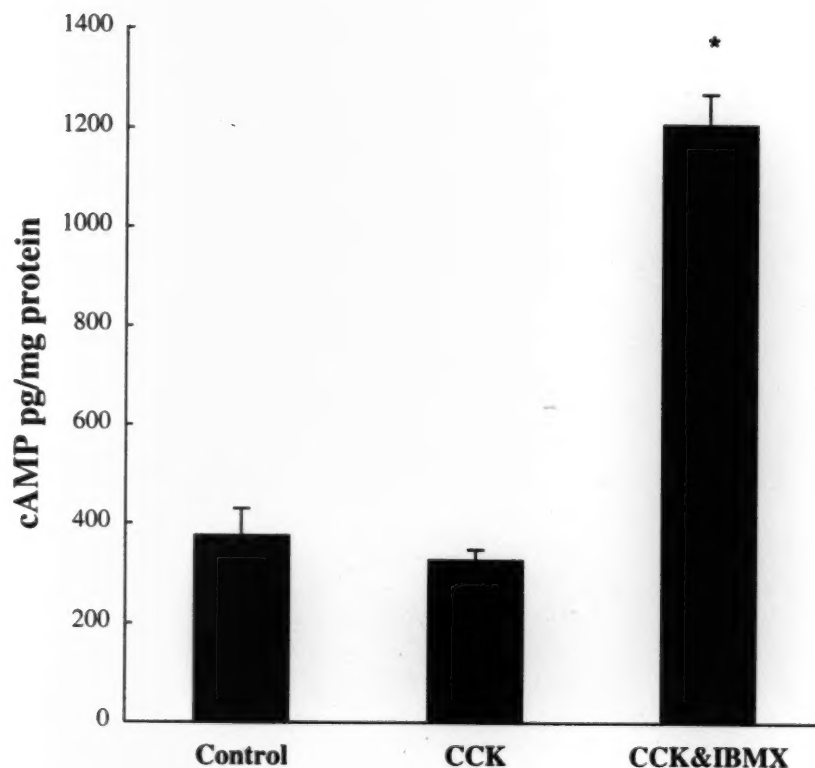


Figure 38. The effect of CCK-8 and IBMX on cAMP accumulation in the dually perfused jejunum. Absorption of labelled 3-O-MG was measured for the duration of the experiment (Table 2). 8 pM CCK-8 was added to the vascular circuit after 30 min of perfusion. After 20 min of perfusion with CCK-8 or CCK-8 and IBMX in the vascular circuit the tissue was removed from the animal, and the cAMP content immediately measured. The control column (steady-state rate bar) was the mean value $\pm$ SEM, $n=4$ same value as in Fig .36 and 37, * indicates significance $P < 0.001$. Results presented are mean values $\pm$ SEM, $n=4$. See Table 2 for rates of 3-O-MG absorption measured in the same tissue.

The cGMP content was also measured in the same samples as for cAMP content. From these experiments (2 sets in triplicate) CCK did not increase cGMP significantly, but 0.1 μ M forskolin increased the cGMP content 3 fold and 1 mM IBMX increased the content by 1.6 fold.

D. Effect of an endothelin antagonist on CCK-8-inhibition of hexose absorption in the vascular and lumenally perfused jejunum

To determine if CCK-inhibition of hexose transport is possibly mediated through the release and subsequent action of endothelin, a non selective endothelin antagonist was used in the dually perfused model. Addition of 0.1 μ M of the endothelin antagonist PD 145065 to the vascular circuit prior to the addition of 8 pM CCK-8 caused a significant inhibition of CCK-8 action on hexose absorption for 3 animals (**Fig. 39**).

Also, the action of CCK-8 on 3-*O*-MG absorption was significantly attenuated when an anti-endothelin antibody was added to the vascular circuit (a 15 % decline from steady-state) which is similar to the result obtained using an endothelin antagonist (**Fig. 39**). The antibody data is preliminary: only one animal tested.

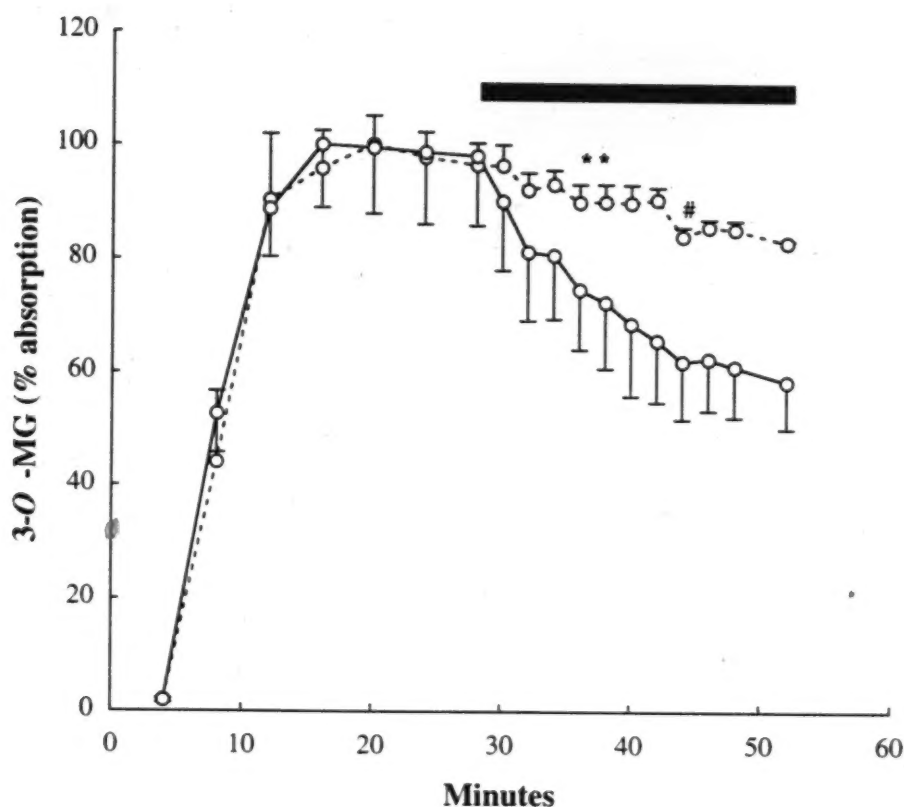


Figure 39. The effect of an endothelin antagonist (PD 145065) on CCK-inhibition of 3-O-MG absorption.

The solid line represents the rate of absorption of 3-O-MG without the endothelin antagonist; 8 pM CCK-8 was added to the vascular circuit at 30 min. (solid black horizontal bar) (Data points are from Fig. 27, $n=5$). The dashed line represents the rate of absorption of 3-O-MG when the tissue is pretreated with 0.1 μ M PD145065, ($n=3$). Results presented are mean values $\pm$ SEM, ** indicates significance $P < 0.001$ from control (solid line), any point following from the same series is significant. # indicates significance $P < 0.001$ from steady state rate.

E. SGLT1 abundance in the mucosal membrane at specific hexose transport rates

To determine if the decrease seen in hexose absorption with CCK present in the vascular circuit parallels a reduction in the number of SGLT1 transporters in the BBM, mucosal scrapings were snap-frozen at specific transport rates and analyzed for SGLT1 abundance using Western blotting. CCK-8 (8 pM) caused a significant decrease in 3-*O*-MG absorption, and decreased the abundance of SGLT1 in the membrane (**Fig. 40**) as compared to the steady-state. IBMX (1 mM) added to the luminal circuit induced a slight increase in SGLT1 abundance, whereas 0.1 μ M forskolin caused a slight decrease, for 4 animals.

There was no significant difference in SGLT1 abundance from the steady-state rate from a dually perfused jejunum and a unperfused segment of jejunum under control conditions.

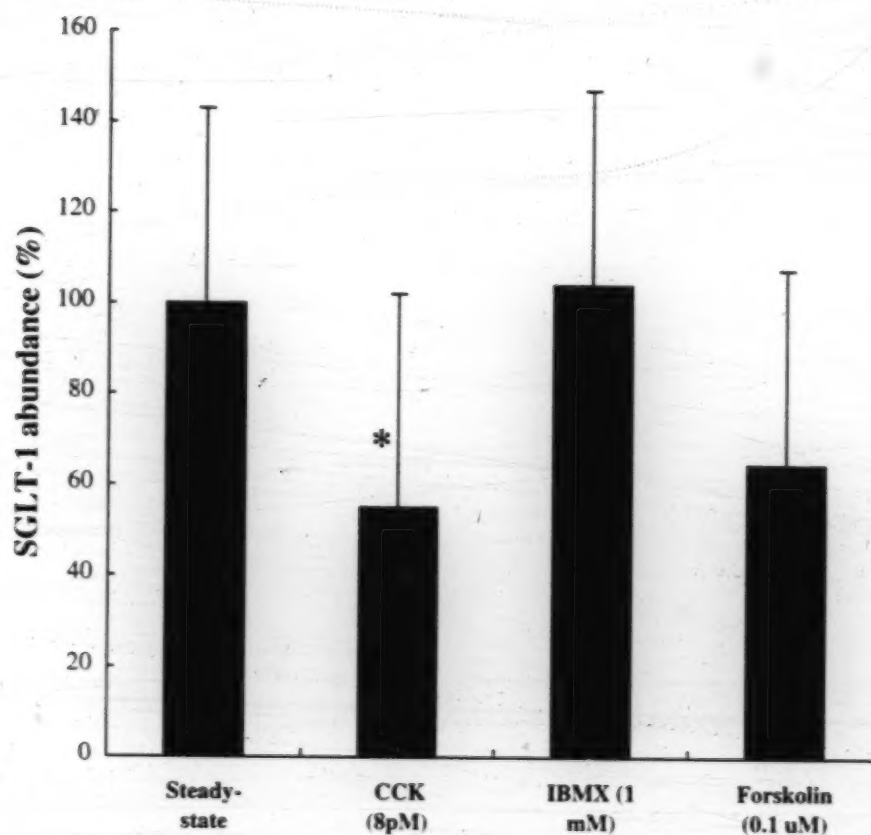


Figure 40. The effect of CCK-8, IBMX, and Forskolin on SGLT1 abundance in the dually perfused jejunum.

Absorption of labelled 3-O-MG was measured for the duration of the experiment (Fig. 41). 8 pM CCK-8 was added to the vascular circuit after 30 min of perfusion. After 20 min of perfusion with CCK-8 in the vascular circuit or 1 mM IBMX or 0.1 μ M forskolin in the luminal circuit the tissue was removed from the animal, opened longitudinally, the mucosal layer scrapped off and immediately snap-frozen in liquid nitrogen. Results presented are mean values $\pm$ SEM, $n=4$ (* indicates $P < 0.05$).

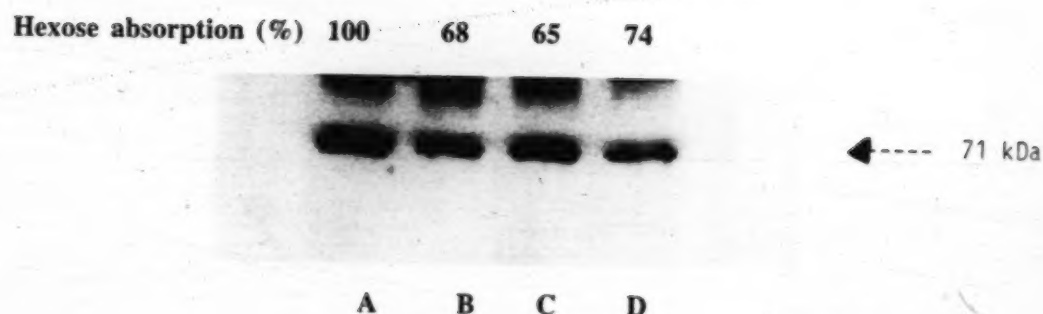


Figure 41. A representative gel showing the effects of CCK, IBMX, and Forskolin on SGLT1 abundance in the BBM at specific hexose absorption rates in the dually perfused jejunum.

After the hexose absorption rate was measured the jejunal segment was excised from the animal, rinsed with Krebs solution, opened longitudinally, and the mucosa scrapped off using a glass slide. The mucosal scrapings were used to make BBM and the SGLT1 protein was visualized using a SGLT1 type antibody. **A.** protein density after 25 min of perfusion with 3-*O*-MG (steady-state rate), **B.** After 20 min. with 8 pM CCK-8 in the vascular circuit. **C.** After 20 min. with 1 mM IBMX in the luminal circuit. **D.** After 20 min. with 0.1 μ M forskolin in the luminal circuit.

F. CCK-8 action on hexose absorption in streptozotocin treated animals

It has been well documented that i.v. injection of streptozotocin will produce an increase in blood glucose concentrations in rats within a couple of days. To determine if the CCK-8-induced acute negative feedback pathway is altered in animals that have chemically induced diabetes, hexose absorption, with and without CCK present, was measured in rats three weeks after streptozotocin treatment (see **Fig. 10**). CCK-induced inhibition of 3-*O*-MG absorption was significantly attenuated in the streptozotocin treated as compared to the untreated animals (**Fig. 42**). To determine the relative rate of 3-*O*-MG absorption in streptozotocin treated animals a correction factor was included in the calculation (**equation 1**) of hexose absorption. The surface area in the lumen has been shown to increase after the animal has been treated with streptozotocin (Thomson, Keelan et al. 1987) (correction in main citation- pg 429 (control chow diet wet weight = 50 mg/cm length)) so the increase in surface area was incorporated into this study. Streptozotocin treated rats are shown to significantly increase the rate of 3-*O*-MG absorption as compared to control (**Table 3**).

3-O-MG absorption ($\mu\text{mol} / \text{g dry wt/ h}$)	3 wk streptozotocin	untreated control animals
at steady state rate (after 25 min)	*125 $\pm$ 13.4	108 $\pm$ 12.7

Table 3. Effect of streptozotocin on 3-O-MG absorption in the dually perfused jejunum.

The absorption rate for control untreated animals, (n=3) for streptozotocin treated (n=5)
Results presented are mean values $\pm$ SEM. * indicates significance $P < 0.05$ from untreated animals.

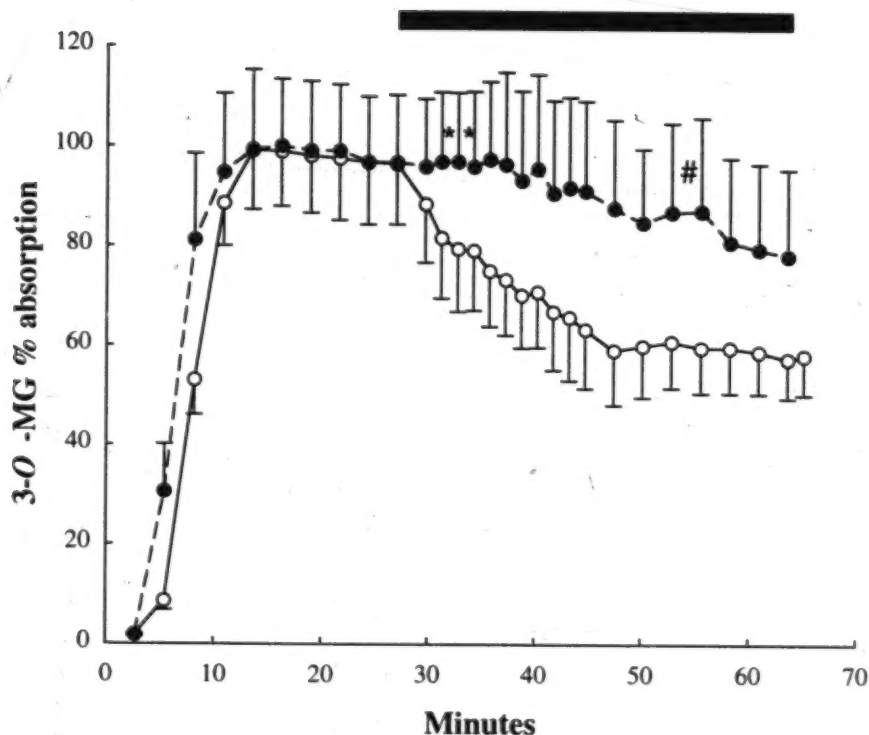


Figure 42. The inhibition of CCK-8 action on hexose absorption in streptozotocin treated rats using the dually perfused technique. CCK-8 was added to the vascular perfusate (horizontal solid black bar) after 30 min. The dashed line (●) represents the 3-O-MG absorption in post three week streptozotocin treated rats (n=5). The (○) solid line represents 3-O-MG absorption from untreated animals (n=3). Results presented are the % absorption mean values $\pm$ SEM. ** indicates significance $P < 0.001$, as compared to the control response, any point following from the same series is significant. # indicates significance $P < 0.05$ from the values at steady state.

A summary of results is provided below to help in clarifying this chapter (Fig. 43).

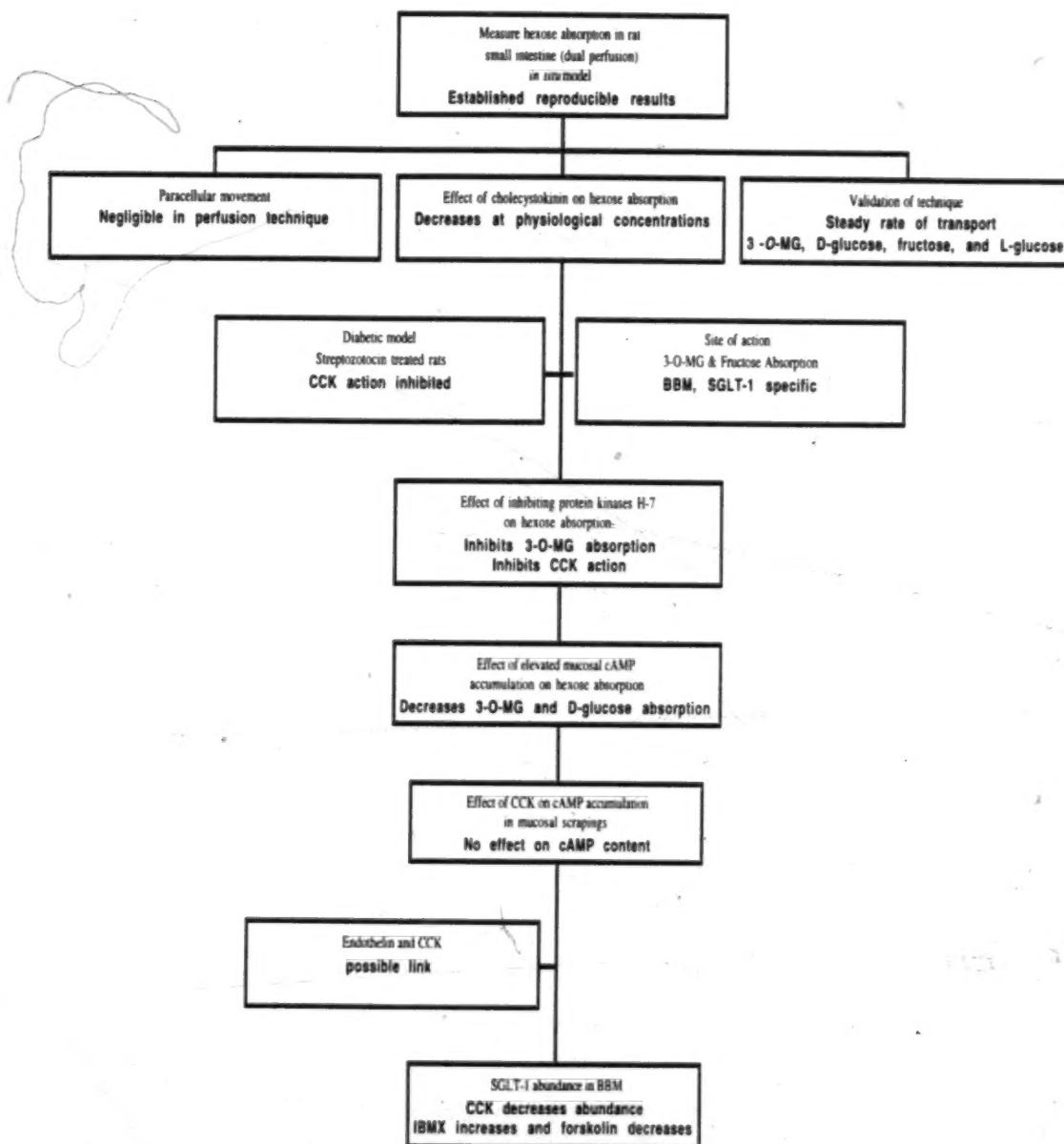


Figure 43. Summary of results.

CHAPTER IV

IV. DISCUSSION

The results from this study suggest the existence of some novel physiological events. The first is the involvement of CCK in acutely regulating the rate of hexose absorption by the small intestine. This rapid CCK-induced reduction of hexose absorption that occurs in the small intestine indicates that the tissue not only can accommodate for various glucose loads in the lumen by distributing absorption along the length, but can also regulate the rate at which it absorbs. This rapid regulatory event therefore puts a greater emphasis on the role this tissue has in maintaining glucose homeostasis. Secondly, CCK appears to act by decreasing the number of hexose transporters in the BBM. Thirdly, the studies of changes in cyclic nucleotide concentration and associated protein kinases in relation to absorption will help clarify the role they play in modulating hexose absorption. And finally, the reduced ability of CCK to regulate the rate of hexose absorption in experimentally induced diabetic animals, suggests this may play a role in this disease.

Hexose transport from the lumen of the small intestine to the vascular bed draining the tissue is apparently regulated by a complex set of interactions between endocrine, paracrine and neural factors. One such factor involved in modulating transepithelial hexose transport in the small intestine is the presence of hexoses in the lumen (Csaky and Fischer 1981; Debnam 1982; Karasov and Debnam 1987). Maenz and Cheeseman (1986) and Karasov and Debnam (1987) have shown that this hexose-mediated regulation is selective for transport across the basolateral pole of the enterocytes and occurs within a four hour period. The signalling pathway involved is, as yet, unclear, although peptide hormones may play a role (Cheeseman and Tsang 1996; Hasegawa, Shirohara et al. 1996). The only evidence associating CCK and carbohydrate absorption is the work of Schwartz and Storozuk (1985) who showed that when CCK-8 was infused into an animal for 14 days, there was a decrease in galactose absorption across the jejunum and an increased absorption

across the ileum. The shift in hexose absorption observed in this study is very difficult to interpret, because the response could be the outcome of an adaptive process or a prolonged regulatory event.

Before discussing the effect of CCK on hexose absorption, there are a few observations that clearly identify the route of hexose absorption across the luminal and vascularly perfused preparation and some key features which have a direct bearing on the results obtained using the dually perfused preparation, which was used extensively in this project.

A. Validation of the dual vascular and luminal perfusion preparation performed *in situ*

The dually perfused preparation used was similar to that used by Bronk and Ingham (1979) and Pennington, Corpe et al. (1994). The vascular and luminal perfusion preparation of the intestine originated from the work of Mond (1924) and was later reassessed and modified by Parsons and Prichard (1968). They identified critical factors that affect the absorption of hexose - both flow rates in the vascular and luminal circuits, and any air bubbles entering the vascular circuit. In all experiments performed in our studies the flow rates of the luminal and vascular circuits were the same and constant, and air was prevented from entering the vascular circuit by using a nylon mesh bubble trap. The rate of 3-O-MG absorption using the dual perfused preparation was 3 fold greater in our preparation than the rates found using frog intestine (Boyd and Parsons 1978), corrected for vascular flow rate and substrate concentration. The variation in transport rate is likely due to species variation and the fact that their experiments were performed at room temperature ie. about 15 °C cooler than in the system I used. However, the steady-state rate of D-glucose absorption I observed using the dual perfused preparation was very similar to that determined by Hanson and Parsons (1976) using rats, $217 \pm 21 \mu\text{mol/g dry wt/h}$ compared to $222 \pm 20 \mu\text{mol/g dry wt/h}$, respectively. Interestingly, the rate of absorption of D-glucose monitored using the dual perfusion model in this study is only half

the rate obtained in the lumenally perfused *in vivo* model. This discrepancy could be the result of a lack of hormonal modulators which are not present in the dually perfused preparation and which have been shown to play a role in increasing glucose absorption in the whole animal (Cheeseman and Tsang 1996).

1. *Hexose transcellular transport in the dually perfused preparation*

The accepted model of carrier proteins responsible for hexose transcellular transport across the enterocyte is depicted in **Fig 4**. To determine if the hexoses used in the dual perfusion model are being transported by these carriers some preliminary experiments were performed. The absorption of 3-*O*-MG was primarily carrier mediated as indicated by 1) the effect of phloridzin in the luminal circuit, the results being similar to that of Boyd and Parsons (1978) and Roig, Vinardell et al. (1993), and 2) the negligible absorption of L-glucose, which is a poor substrate for SGLT1 (Bronk and Ingham 1976). One variable that could affect transcellular substrate absorption is the metabolism of that substrate by the enterocytes. Where necessary 3-*O*-MG, a nonmetabolizable analogue of D-glucose that uses the same carrier proteins as D-glucose, was employed.

The vectorial transfer of fructose from the lumen of the intestine to the vascular bed occurs by two separate Na⁺-independent facilitative transport proteins in series: GLUT5, located in the BBM, and GLUT2, found in the BLM. It has been suggested that the rate of absorption of this ketose is comparable to the rates of glucose and galactose uptake (Wright, Hirayama et al. 1994). However, Holloway and Parsons (1984), using a vascular perfused preparation, found the rate of D-glucose absorption to be 1.5 fold greater than that of fructose. Also, my results from the dually perfused preparation showed glucose absorption was 6.8 fold greater than that of fructose, and 3-*O*-MG absorption was 3.2 fold greater. The lower ratio found by Holloway and Parsons (1984) is likely due to the different substrate concentrations employed in these two studies. The results of these

two studies suggest that the jejunum absorbs glucose and galactose at a faster rate than fructose. The fact that fructose entry into the enterocyte is not driven by the Na^+ gradient and the driving force being rather speculative, either being purely concentration driven or by the metabolism that occurs in the enterocytes are the possible explanations for a reduced absorptive rate. Furthermore, it is likely that the metabolic fate of fructose is one of the primary determinants in regulating absorption and therefore a species dependent phenomenon depending on the animals ability to metabolise fructose (Bismut, Hers et al. 1993).

The rate of absorption for D-glucose using the dual perfusion preparation was approximately 2.1 fold faster than the rate seen when using 3-O-MG, at equimolar concentrations. Since 3-O-MG is transported by the same carrier proteins as D-glucose the differences seen in the rate are likely due to differences in the affinity of the two substrates for the BBM carrier where D-glucose has a much greater affinity for SGLT1 than does 3-O-MG (Ikeda, Hwang et al. 1989). Also, the removal of a metabolic fuel for the absorptive cell could indirectly affect the Na^+/K^+ ATPase and in turn shift the intracellular Na^+ concentration which is concomitant with changes in: 1) the membrane potential which has been shown to modulate the SGLT1 (Kimmich 1990), and 2) the intercellular spaces between the enterocytes which in turn play a significant role in regulating nutrient absorption across the intestinal epithelium (Boyd, Cheeseman et al. 1975).

2. *Importance of tissue oxygenation in the dual perfusion model*

In order to help determine limitations of the dual perfused preparation, and to verify established characteristics of hexose absorption preliminary experiments were performed which revealed that if the vascular circuit were not established within seconds the constant rate of hexose absorption (Fig. 12) was not seen. Instead the rate would rapidly dwindle to a negligible level within 10 min after the substrate appeared in the vascular effluent. This

decrease is similar to that seen if no bubble traps are included in the preparation (Parsons and Prichard 1968), indicating that 1) the tissue is very sensitive to hypoxia, and 2) leaving stagnant blood in the vasculature, over short periods, can cause spurious results. Hanson and Parsons (1976) used washed erythrocytes in their preparation to aid in maintaining proper tissue oxygenation. The similar transport rates seen in our preparation when whole blood replaced the Ficoll based Krebs solution indicates that this technique provided sufficient oxygen to maintain adequate cellular viability for hexose absorption.

The significance of cellular respiration in the dually perfused model was indicated by a 91% decrease in hexose absorption seen when KCN was included in the perfusate (Fig. 14). The similar 3-O-MG absorptive rates seen when whole blood was vascularly perfused instead of the Ficoll based Krebs, with and without phloridzin present in the luminal perfusate, indicates that the Ficoll based Krebs solution is not hindering the rate of hexose entry into the vasculature.

3. *Paracellular movement and the dually perfused preparation*

The most convincing data that challenges the paracellular route for solute absorption was the combination of a high 3-O-MG phloridzin-sensitive flux and a very low phloridzin-insensitive L-glucose flux (Fig. 12). This strongly suggests that under the conditions of the dual perfused preparation the paracellular route played a negligible role in hexose absorption. This finding of very low paracellular movement is in agreement with the work of Fine, Santa Ana et al. (1993), who found that, *in vivo*, 95% of the luminal glucose load was absorbed by stereospecific, carrier mediated transport. A possible reason for not observing a non selective paracellular movement was that the substrate concentration used in the present experiments never exceeded physiological concentration levels found in the rat small intestine (Ferraris, Yasharpour et al. 1990). This suggests that paracellular movement may only occur at supraphysiological concentrations and is therefore an unlikely

physiological property of this epithelium. The only evidence which suggested a paracellular-type movement was the abnormal absorption of fructose after the cells' oxidative pathway was inhibited using KCN (**Fig. 14**), and when the vascular cannula was not inserted immediately following ligation of the aorta. In each instance the absorption fell to a very slow rate and then after approximately 10 to 15 min. increased steadily. In the case of fructose, absorption would far exceed the steady-state rate of transport seen in all other experiments. Interestingly, the majority of articles supporting paracellular movement use an *in vitro* model where there is a greatly reduced vascular gradient and the tissue would suffer hypoxic damage. By monitoring data from preparations in which the insertion time of a cannula into the superior mesenteric was delayed the absorption rate would obtain a maximum rate very similar to the maximum rate when the cannula was inserted quickly but the rate would rapidly decline to a very slow rate and in some cases would increase after a period of time similar to that which was seen when KCN was added. This suggests that paracellular movement occurs only after severe hypoxia, and is a consequence of damage to the epithelial wall.

One other noteworthy observation is the small component of absorption that is phloridzin insensitive. By examining **Fig. 12** it can be seen that 13 % of 3-*O*-MG absorbed is not phloridzin sensitive. The rate of this component of absorption is not dissimilar to that which was seen by Parsons and Prichard (1968), who found 20 % to be phloridzin insensitive, Roig, Vinardell et al. (1993) found 30 %, and Pennington, Corpe et al. (1994) showed 13 % to be phloridzin insensitive. The results of the phloridzin study, taken together with the L-glucose study, strongly suggest that the remaining component is not by paracellular movement or by diffusion. The remaining small phloridzin insensitive component can therefore be explained by one or a combination of three theories 1) SGLT1 is not completely inhibited by phloridzin, 2) the BBM facilitative carrier GLUT5 is allowing hexoses across other than the preferred monosaccharide, fructose, or 3) that another transporter which is as yet uncharacterized, is located in the BBM.

By identifying the variables that are crucial in maintaining a steady rate of hexose absorption in the dually perfused preparation, and establishing the pathway involved in hexose absorption therefore this technique allows a detailed study on substances that acutely regulate hexose absorption.

B. CCK and hexose absorption

The addition of CCK to the vascular bed in the dual perfusion preparation produced a rapid decrease in the rate at which substrates specific for the SGLT1 appeared in the vascular bed. This decrease could be the result of a nonspecific or more of a general effect modifying the Na^+ transepithelial gradient, or altered vascular flow to the epithelium and therefore would be classified as a nonspecific effect on absorption. The studies from both the L-leucine absorption (**Fig. 23**) and fructose absorption (**Fig. 28**) experiments indicated that the effect of CCK on absorption in the small intestine is specific in two ways: one, specific for the hexose it regulates - only the SGLT1 transporter and not the amino-acid transporter for L-leucine, and two, specific in regards to the type of mechanism - a change in rate of absorption, not a nonspecific type mechanism such as a change in vascular flow, or a change in the Na^+ gradient. These nonspecific actions were disregarded since CCK had no effect on L-leucine and D-fructose absorption, whereas it did affect 3-O-MG and D-glucose absorption. Both the absorption of D and the L isomer of glucose was measured concurrently when determining the IC_{50} of CCK (**Fig. 19**). No significant decrease of CCK-induced L-glucose absorption was monitored, which suggests that, CCK's action is purely on a carrier mediated event.

To determine the site of action of CCK in the absorptive epithelium two methods were used, compartmental analysis of D-glucose washout, and concurrent 3-O-MG and fructose absorption. Washout studies have previously been used successfully to indicate where a rate limiting step occurs for transport across the enterocyte (Boyd and Parsons 1978; Boyd

and Parsons 1979; Cheeseman 1981). In the washout studies, the addition of CCK to the vascular perfusate caused a significant decline in the steady state rate and also in the initial pool size within the epithelium. This effect is similar to that of phloridzin which is known to act specifically on SGLT1 (Boyd and Parsons 1978). This implies that the rate limiting step for the effect of CCK is at the BBM. Furthermore, CCK had no effect on fructose absorption (Fig. 27). Fructose enters across the BBM using a separate hexose carrier from the other monosaccharides, yet it can exit across the BLM using the same facilitative transporter as D-glucose and D-galactose (Fig. 44 below). This supports the view that CCK acts on entry across the BBM and not on exit across the BLM. One unlikely event is that CCK could change the affinity of GLUT2 for specific substrates in the BLM. However the fructose and 3-O-MG absorption data clearly indicated that CCK, while having a significant effect on 3-O-MG, did not affect the rate of fructose absorption. Taken together, the compartmental analysis results and the unaffected fructose absorption indicate that CCK's action is on limiting the entry of aldoses across the BBM i.e. substrates specific for SGLT1.

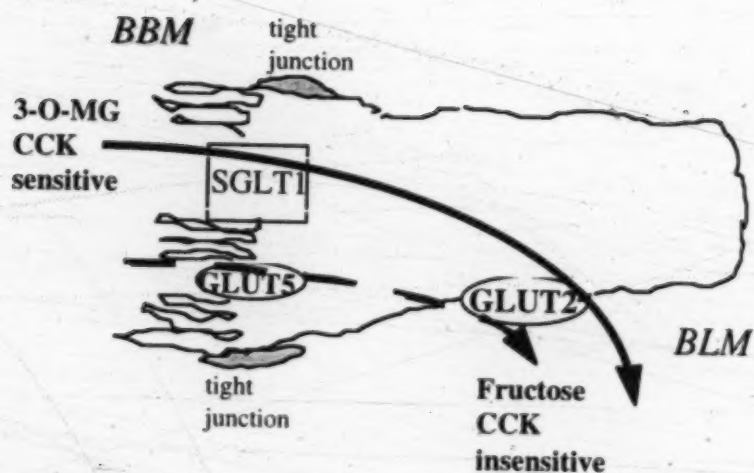


Figure 44. Diagram of hexose transepithelial pathways in the enterocyte indicating loci of sensitivity to CCK. Dashed line represents fructose absorption, solid line represents 3-O-MG absorption. The tight junctions represent the area of attachment to neighboring cells and the area to the right of the tight junction represents the BLM.

A metabolic pathway does not seem to be involved in the CCK-induced reduction of hexose absorption as indicated by comparing the effect on both the nonmetabolized analogue, 3-*O*-MG and the metabolizable D-glucose, **figures 18 and 20**, respectively.

The mechanism whereby CCK causes decreased hexose absorption, whether acting as a classical hormone or through a paracrine route, has not been addressed in this study. CCK is produced by I-type cells which are found close to the enterocytes in the jejunum and therefore has the capacity to act as a paracrine agent, and in so doing avoid the breakdown which occurs during hepatic transit (Doyle, Wolfe et al. 1984). On the other hand the response seen with CCK present in the vascular circuit using the dually perfused preparation occurred within the physiological concentrations found in the rat. Also, the fact that the route used by the peptide in our preparation is the same as that found *in vivo* suggest that it is acting as a classical hormone. As mentioned earlier the IC₅₀ was on the low side of the plasma concentrations and one would assume that it would be greater if acting through a paracrine system.

The physiological plasma concentration of CCK in rats is 0.5 pM in fasted animals and increases postprandially within 15 min to 6 pM, (Liddle, Goldfine et al. 1984). CCK was shown to inhibit jejunal 3-*O*-MG transport with an IC₅₀ of 1.8 pM, i.e. at the low end of the physiological concentration range. Thus, even between meals there would be sufficient CCK circulating in the plasma to significantly inhibit hexose absorption. This suggests that CCK-8 could be acting as a tonic brake on hexose absorption, down-regulating the absorptive mechanism between meals. Subsequently, during a meal the small intestine could be under a stronger influence to decrease its absorption because of the elevated CCK plasma concentration. A possible role for this CCK inhibition seen in the small intestine during a meal is that it is acting as part of a rapid negative feedback mechanism to help regulate blood glucose levels. However, it must also be borne in mind that there are stimulatory mechanisms at work during a meal (eg. GLP-2) and the overall rate of

absorption will be an integrated response to these different factors (Dempster and Kellett 1992; Sharp and Debnam 1992; Pennington, Corpe et al. 1994; Cheeseman and Tsang 1996). Also, because both D and L-type sugars were measured concurrently when determining the IC₅₀ of CCK (Fig. 19), and the results where no significant CCK-induced L-glucose absorption decrease was monitored, and knowing that 3-O-MG is absorbed by carrier mediated transport suggests that, CCK's action is on transcellular transport pathway.

From our results CCK has been shown to decrease the rate of hexose absorption across a segment of rat jejunum significantly. However, the effects of increasing plasma CCK levels in humans was shown to have no inhibitory action on plasma glucose concentration after a bolus of glucose was given intralumenally (Liddle, Rushakoff et al. 1988). The effect of CCK on glucose absorption could have been masked by the stimulatory effect of GLP-2 which would upregulate glucose transport. Also, I have shown that the concentration of CCK is critical regarding the actions on intestinal absorption, therefore the concentration in their experiments could have been outside the activity range for decreased hexose absorption to occur. One other explanation regarding the observed differences is that there is species variation regarding the actions of CCK in rats and humans.

C. CCK's effect on SGLT1 abundance

The maximal degree of inhibition for D-glucose was 45 %, and for 3-O-MG, 41 %. A question that can be raised from these results is the issue of why the effect is only moderate and not a more complete inhibition. From a physiological point of view it makes sense that carbohydrate absorption cannot be totally suppressed in the small intestine because of the unpleasantities associated with sugars reaching the large intestine, and the need for carbohydrates to maintain the basic metabolic requirements and requirements from the central nervous system. The limited decrease in transport produced by CCK in the vascular

perfusate at concentrations ranging from 1 pM to 8000 pM could result from either a receptor mediated or a post receptor event. The CCK receptors, once bound, have been shown to undergo a rapid desensitization (Roettger, Rentsch et al. 1995) and therefore prolonged or intensive biological effects are attenuated (Abdelmoumene and Gardner 1980). This receptor-mediated event has been demonstrated in the CHO cells expressing CCK-A receptors (Roettger, Rentsch et al. 1995). A post receptor event is likely involved, as suggested by the decrease seen in SGLT1 abundance with CCK present in the vascular perfusate (Figs. 40 and 41). The significant decrease in abundance seen within 20 min after CCK addition to the vascular circuit indicates that this peptide is involved in a rapid post translational event regarding SGLT1 abundance and could likely mean that it regulates translocation in a manner similar to that of GLUT1 and GLUT4 (James, Brown et al. 1988). Results that support this type of regulation are: changes that occur in surface area in enterocytes when glucose absorption is increased with epidermal growth factor (Hardin, Buret et al. 1993), and recently by showing GLP-2 increasing SGLT1 abundance in this tissue (Cheeseman 1997). There is also some evidence using *Xenopus* oocytes expressing SGLT1 that protein kinase A and C mediate exo and endocytosis, respectively, of vesicles containing SGLT1 (Hirsch, Loo et al. 1996). Because the action of CCK occurs at the BBM and the peptide is added to the vascular circuit suggests that the effect is mediated by a cytosolic second messenger and does not occur directly through a receptor mediated endo or exocytosis. The CCK-induced decreased abundance seen in this study could result from a trafficking phenomenon by decreasing insertion of SGLT1, increasing removal from the BBM, or offsetting a possible recycling step. However, the decrease in SGLT1 abundance indicates that the action is not likely to be mediated through the proposed regulatory subunit of SGLT1 (RS1), unless it acts as a chaperone (Veyhl, Spangenberg et al. 1993).

D. CCK receptors involved in hexose regulation

In an effort to identify the receptor type mediating the CCK response we used cholecystokinin tetrapeptide (CCK-4), a CCK-B agonist and CR1409 a type A receptor antagonist. Gastrin-17-I has been used as a selective agonist to distinguish between the two CCK receptor subtypes (Yu, Noguchi et al. 1987; Huang, Yu et al. 1989), although it is not entirely specific for the B receptor. However, CCK-4 is believed to be very specific for the B receptor which makes it a very useful agonist for distinguishing between the two receptor sub-types (Jensen, Qian et al. 1994). We used a broad concentration range of CCK-4 and found only a small decrease in 3-O-MG absorption when the peptide was added to the vascular circuit prior to or after CCK-8 administration (**Fig 21**). This would support the view that CCK-B receptors are not significantly involved in this response. In contrast, the CCK A-type antagonist CR-1409 has been shown to be a potent (Makovec, Bani et al. 1986) and specific antagonist for the CCK-A receptor (Jensen, Qian et al. 1994). In these experiments CR-1409 almost completely blocked the action of CCK-8 (**Fig. 22**). These two sets of data indicate that this action of CCK is mediated by the A-type receptor. In addition, CR-1409 added alone to the vascular perfusate produced no increase in 3-O-MG absorption, indicating that there was little, if any, endogenous CCK modulating hexose absorption in the dually perfused preparation. Together, these results strongly suggest that the action of CCK-8 in the jejunum is mediated via a CCK-A type receptor, which is also known to be present in brain (Hill, Shaw et al. 1990), gallbladder (Shaw, Madac et al. 1987; Schjoldanger, Molero et al. 1989), pancreatic acinar cells (Louie, May et al. 1986; Jensen, Qian et al. 1994), and islets of Langerhans (Sakamoto, Goldfine et al. 1985; Verspohl, Ammon et al. 1986). The fact that CCK, when added to the vascular perfusate, produces the effect supports the theory that an A-type receptor would be located on the BLM. The binding studies performed on BLMVs did not provide any conclusive evidence for an A type receptor on the basolateral membrane of the enterocyte, and therefore do not permit any conclusions to be made regarding the exact site

of the CCK-A receptor. More experiments are needed to confirm whether or not A-type receptors exist on the BLM of the enterocytes. A possible explanation for the unsuccessful binding study was that in the preparation bovine serum albumin was included and only after these studies was it shown that bovine serum albumin greatly affects CCK binding (Huang, Talkad et al. 1995). Furthermore, the effect of CCK action was inhibited by pretreating the tissue with PD 145065, a nonselective endothelin antagonist in the vascular perfusate (Fig. 39) and the preliminary experiment with endothelin antibody which suggests that CCK's action could be mediated through the release and subsequent action of endothelins. This possible mechanism will be discussed further below.

E. Possible signalling pathways involved in mediating CCK action

A cAMP mediated regulatory pathway of transepithelial hexose transport in the mammalian small intestine was proposed when cAMP accumulation was shown to change electrolyte transport in this tissue (Field 1971; Field 1972). Shortly thereafter, acute regulation of SGLT1 via the adenylyl cyclase pathway in intestinal absorptive epithelium was investigated using isolated enterocytes (Moreto, Planas et al. 1984; Kimmich, Randles et al. 1985; Sharp and Debnam 1994), and isolated membrane vesicles (Murer 1980; Sharp and Debnam 1994). How increased levels of cAMP produced a parallel increase in glucose accumulation from the *in vitro* studies is controversial, theories include increasing the rate of entry (Murer 1977; Murer 1980; Moreto, Planas et al. 1984; Tai 1986; Sharp and Debnam 1994), increasing the membrane potential, which could also increase uptake (Sharp and Debnam 1994), or inhibiting exit (Holman 1975; Holman 1976; Randles and Kimmich 1978) and therefore allowing an increase in glucose accumulation with the net result being a decrease in hexose absorption. The results, obtained using 8-Br-cAMP, forskolin, and IBMX, in this study indicated that the adenylyl cyclase pathway is involved in acute regulation of glucose absorption in the small intestine. In support of this statement

are the results from **Figs 34, 37 and 35** which showed these compounds when added to the luminal perfusate caused significant inhibition in 3-*O*-MG absorption. Also, the *in vivo* results **Fig 33** showed 8-Br-cAMP, when added to the luminal perfusate, caused a decline in D-glucose absorption. Furthermore, forskolin **Fig. 37** and IBMX **Fig 36** both increased the cAMP accumulation in tissue which had hexose absorption declining.

The reasoning for linking the CCK-induced hexose decrease with a change in cyclic nucleotides was due to 1) The work by Sharp and Debnam (1994) who showed that changes in cAMP accumulation was associated with changes in hexose transport, 2) the CCK receptor belonging to the R7G family, and these receptor proteins are known to commonly use adenylyl cyclase as their effector (Strosberg 1991), and 3) CCK's ability to activate adenylyl cyclase in pancreatic tissue (Williams and Blevins 1993). Evidence to support a link between CCK action on hexose absorption and cAMP included the similarities in the results showing a net decrease in hexose absorption when including 8-Br-cAMP, IBMX, or Forskolin in the luminal circuit in the dually perfused preparation. Also, the loci of 8-Br-cAMP action was identical to that of CCK, as indicated by the unaffected fructose absorption i.e. BBM. And finally, the attenuated CCK response when inhibiting protein kinases with a nonspecific kinase inhibitor (H-7) added to the luminal circuit prior to the addition of CCK. However, the definitive result which ruled out CCK involvement with cAMP was that the cyclic nucleotide accumulation did not increase with CCK present in the vascular perfusate, whereas IBMX and Forskolin did increase the cyclic nucleotides, and all caused a significant decline in hexose transport. This eliminates any possible link between CCK and cAMP or cGMP pathways regarding hexose transport. These results do not indicate if other protein kinases could be involved in CCK-induced inhibition of hexose transport, but do suggest that protein kinase C may also play a role in regulating hexose absorption (**Fig. 31**), and due to the effect of the kinase inhibitors (**Figs 30 and 31**) support a regulatory role of kinases on hexose absorption.

A concern regarding the cAMP mediated effect on hexose absorption was the fact that an increase in cAMP in I-type cells has been shown to stimulate the secretion of endogenous CCK (Barber, Walsh et al. 1986; Koop and Buchan 1992). Therefore, the decrease in hexose absorption obtained using compounds that increased cAMP accumulation could be the result of endogenously released CCK from I type cells found in the dually perfused preparation via increases in cAMP. The only evidence which argues against such a response is the differences seen in SGLT1 abundance in the presence of CCK or IBMX: IBMX produced no significant decrease in SGLT1 abundance, whereas CCK did.

The fact that CCK did not increase cAMP accumulation, yet a chemically induced rise in cyclic nucleotides showed actions similar to those of CCK indicates that cyclic nucleotides have a potentially important role in modulating hexose absorption. However, these data suggest that CCK does not mediate its actions through cyclic nucleotides, and therefore two separate mechanisms may be involved in acutely decreasing SGLT1 activity in the dually perfused jejunum. Thus, any receptor that is linked to the adenylate cyclase pathway or a pathway which in turn can modify cyclic nucleotide concentrations would likely have a significant influence on hexose absorption across the small intestine.

F. CCK and hexose absorption in experimentally induced diabetic animals

The decision to include studies on experimental diabetes was to determine if the CCK-influenced hexose decrease, seen in non diabetic rats, is altered. If the CCK-hexose absorption pathway is altered the small intestine could allow unregulated amounts of hexose into the bloodstream, causing hyperglycemia, which is commonly seen in diabetics. It would not be unlikely that an alteration in CCK is involved in diabetes as other gastrointestinal peptides were shown to play a significant role in regulating glucose homeostasis indirectly through the entero-insular axis, namely GIP (Turner, Etheridge et al. 1974) and GLP1 (Schmidt, Siegel et al. 1985). Direct evidence which links

hyperglycemia seen in diabetics to CCK was the study where plasma CCK levels were significantly lower in type II diabetics after feeding compared to controls (Rushakoff, Goldfine et al. 1993). These results suggests, that a lower concentration of CCK and thus a decrease in the negative feedback pathway in hexose absorption across the small intestine could be one of the contributing factors of hyperglycemia.

We used the streptozotocin treated rat as a model for diabetes. The mechanism involved in the streptozotocin- induced hyperglycemia is postulated to be a selective destruction of the B cells in the pancreas (Inoue, Norgren et al. 1997) which, in turn, decreases the output of insulin and greatly reduces the actions of this hormone on the liver and peripheral tissues with the end result being excess glucose in the systemic circulation. We defined diabetic animals as those with a plasma glucose concentration > 300 mg/dl. A characteristic of these animals was the increased absorptive rate of 3-*O*-MG, similar to that reported by Csaky and Fischer (1981). They hypothesized that the increase in 3-*O*-MG absorption was the result of the chronic hyperglycemia which would cause an increase in the number of transport proteins in the BLM. The effects of diabetes on SGLT1 are an increase in mRNA (Miyamoto, Hase et al. 1991), increasing expression of SGLT1 (Fedorak, Cheeseman et al. 1991; Kurokawa, Hashida et al. 1995), and increasing the functional SGLT1 protein nearer to the crypt than in normal rats (Debnam, Smith et al. 1995). From my studies no conclusions can be drawn on how the increased transport was induced.

What is novel in this study is the reduced sensitivity to CCK. CCK (8 pM, the maximal effective concentration) when added to the vascular perfusate decreased the absorptive rate $t_{1/2} = 28.4 \pm 0.2$ min compared to control 11.3 ± 0.1 min, and only by 20 %, compared to 41 %, in controls. These results are preliminary and further studies are needed to identify if the desensitization is a receptor mediated event or that the possible second messenger system involved in the CCK/(endothelin?) action has not been disrupted. Also, it would be of interest to see if the inhibition is at the BBM as was seen with CCK in normal rats. The fact that streptozotocin was administered three weeks prior to the hexose absorption studies

and the effects of streptozotocin on the intestinal tissue is unknown, one cannot rule out that streptozotocin and not the physiological changes that occur due to streptozotocin are part of reasons for the alteration seen in hexose absorption in chemically induced diabetes.

G. CCK and possible secondary mediators

Because the dual perfusion preparation of the small intestine does not disrupt the normal tissue geometry, and because no evidence of CCK receptors was found on the BLM of enterocytes we investigated some of the potential interactions between neural and endocrine tissue on the CCK-induced modulation of hexose absorption. The intramural nerve network is very extensive in the submucosa of the small intestine and CCK has been shown to act on primary afferent fibers in rats (Forster, Green et al. 1990). Therefore, to help establish if a neural component is involved in any CCK action, the Na⁺ channel blocker tetrodotoxin (TTX) was used as an inhibitor of neural activity. CCK has been shown to be a neurotransmitter, as well as a hormone, which raised the possibility that the infused peptide could be acting on receptors making up part of a neural control loop. Therefore, adding TTX to the vascular perfusate would indicate if this sodium channel blocker could abolish or reduce the inhibition produced by CCK-8. The results, showing a failure to block the CCK effect with this agent, (Fig. 24), suggest that CCK is acting downstream from any neural pathway (Dakka, Cuber et al. 1993). However, we cannot overlook the possibility that a nonadrenergic-noncholinergic pathway could be involved. Also, the rate of 3-O-MG absorption in the absence of CCK was not affected by TTX, suggesting that the TTX sensitive intramural nerves do not play a role in regulating the steady-state rate of hexose absorption under these conditions.

The intestinal peptide somatostatin (SS) has also been hypothesized to regulate the rate of nutrient uptake in the intestine (Unger, Ipp et al. 1977). It has been shown that CCK-8-induced activation of antral and fundic D cells can cause the secretion of SS (Lloyd,

Maxwell et al. 1994), so we also tested for SS involvement in the CCK-induced regulation. Both SS-14 and 28 are found in neural and endocrine tissue in the intestine (Keast, Furness et al. 1984), but no effect was seen on the absorption of 3-*O*-MG when either peptide was added to the vascular circuit (**Fig. 25**). This would suggest that the action of CCK is not mediated via the release of SS.

The possibility that endothelins are linked to the CCK-induced decrease in hexose transport was deduced from our results where we showed a rapid decrease in absorption, and that of (Kuhn, Fuchs et al. 1997) where they showed that endothelin -1 (ET-1) when added to the circulating bath caused a rapid decrease in glucose absorption in human jejunal segments with a similar time of action as that for CCK. Also, the dual perfusion model that we used contains the tissue responsible for ET-1 release (Hickey, Rubanyi et al. 1985) and binding of endothelin has been demonstrated in the GI tract (Rubanyi and Polokoff 1994). From the studies using the nonspecific endothelin antagonist, where a significant attenuation in the action of CCK on hexose absorption was observed, suggests that CCK could be mediating its action via the endogenous release of endothelins (see **Fig 39**), or that inhibiting endogenously produced endothelin masks the effects of CCK in this preparation. One easy way to determine if endothelins are involved would be to include them in the perfusate, but because of their strong vasoconstrictor ability would hinder any measure of vascular appearance leading to problems in interpretation. It is of interest that both TTX and the somatostatins showed no effect on 3-*O*-MG absorption, yet the addition of endothelin antagonist or antibody to the vascular circuit caused a significant inhibition in CCK action which was similar to the effects of H-7.

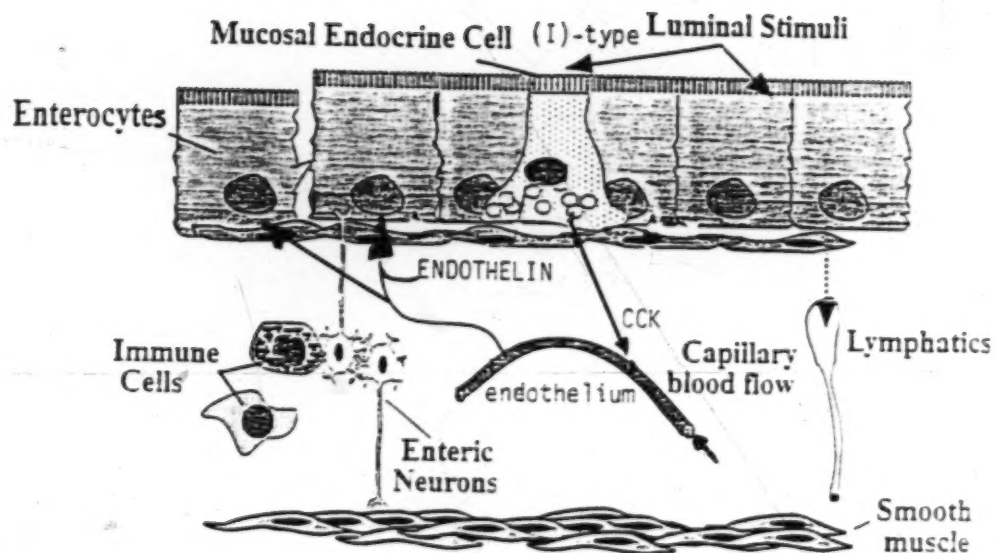


Figure 45. A diagram showing a possible route of action for CCK/endothelin on hexose absorption in the dually perfused jejunum. Modified from Chang and Rao, (1994)

H. Physiological significance of CCK in the regulation of hexose absorption

The intestinal epithelium represents the largest cellular interface between the external environment and the internal environment, and constitutes the major barrier through which molecules can either be absorbed or secreted. Therefore, it is obvious that if the absorption of a primary nutrient, such as carbohydrates, is acutely regulated it would then effect the homeostasis of carbohydrate in the organism.

As mentioned earlier, the transport of glucose across the small intestine is regulated by a complex set of endocrine, paracrine and neural interactions. From the studies with CCK it is not possible to indicate clearly which of the three pathways it is acting through, but what the results clearly indicate is that CCK is involved in a negative feedback pathway, not TTX sensitive, which can rapidly decrease hexose absorption across the jejunum. Furthermore, this CCK-induced decrease is associated with a decrease in the abundance of SGLT1 protein found in the BBM. These findings suggest that the small intestine plays a significant role in controlling the rate of supply of glucose to the systemic circulation. The slowing of the rate of glucose absorption in the jejunum means that the ileum will be important for ensuring complete absorption of carbohydrate. Booth (1961) said: 'if a load greater than the capacity of the jejunum is experienced then the ileum will absorb the remainder acting as a functional reserve'. One study which showed a chronic effect of CCK-8 administration on hexose absorption demonstrated that the absorption across the jejunum of the animal decreased, whereas the absorption across the ileum increased (Schwartz and Storozuk 1985). Interpretation of these results combined with our findings of an acute CCK-induced decrease in hexose absorption suggest that the ileum, being downstream from the jejunum, would receive a greater load of hexose due to the reduced absorption in the jejunum caused by CCK, and over the 2 week period of elevated plasma CCK levels the ileum would increase its capacity to absorb the hexose load by adaptive

mechanisms (Booth 1961). This CCK-induced decrease in absorption can further explain some of the complications seen in patients with extensive ileal resection, where the jejunum is acting under the influence of CCK and reducing carbohydrate absorption, with the end result being a build up of sugars in the large intestine. This problem is likely compounded due to the removal of other endocrine cells which secrete stimulators of glucose absorption (Cheeseman 1997).

By a combination of its satiety effect (Gibbs, Young et al. 1973; Gibbs and Smith 1977), delaying gastric emptying (Liddle, Morita et al. 1986; Liddle, Rushakoff et al. 1988), decreasing transit time in the small intestine (Bertaccini and Agosti 1971; Levant, Kun et al. 1974) and the decrease in the rate of glucose absorption by the small intestine shown here, these results indicate that CCK plays a very significant role in lowering plasma glucose levels or stopping them from rising too high.

The similar actions of increased cAMP accumulation in the mucosa on hexose absorption and that of CCK emphasises the importance of this physiological response. It is possible other gastrointestinal peptides or neurotransmitters could act through the adenylate cyclase pathway and provide another negative feedback control system for glucose absorption.

One other noteworthy point was the specificity of action of CCK, it only affects SGLT1 in the BBM. [What was deceiving was that increasing the cAMP concentration had a similar effect to CCK.] The likelihood that I would have stumbled upon the only carrier protein in this tissue that can undergo this type of rapid regulation is very unlikely and therefore suggests that other transporters could endure the same fate as that of SGLT1 and the actions of CCK. The absorptive cells lining the small intestine are equipped with a huge number of carrier proteins for the various nutrients (amino-acids, dipeptides, and nucleosides, etc.) to be absorbed. These findings therefore indicate that transport physiology in the small intestine, regarding acute regulation, has substantial opportunities for future investigators.

I. Conclusions and future directions

The ability of CCK to regulate hexose absorption acutely in the small intestine has been clearly demonstrated. Its action is mediated primarily through CCK-A type receptors possibly causing the release of an endothelin. The endothelin would then cause a decrease in the abundance of SGLT1 which decreases the amount of substrate taken across the BBM, with the net result being a decrease in glucose and galactose absorption. It is obvious that more studies are needed to help identify the exact location of the A-type receptor, including specific bovine serum albumin concentrations and possibly incorporating cibacron blue to enhance agonist binding (Yuan, Wank et al. 1993) could help to identify if the receptors exist on the BLM of the enterocyte. The signalling pathway involved in the CCK-induced hexose decrease has not been clearly established, instead what is shown is that elevated cAMP concentrations mimic the effect of CCK and are equally potent and capable of decreasing hexose absorption, but possibly through a different mechanism from that described for CCK.

The establishment of the existence of a rapid negative feedback pathway for controlling hexose absorption in the small intestine extends the functionality of the tissue regarding glucose homeostasis. Instead of an immediate and rapid absorption of all SGLT1 specific substrates there is a slowing in the rate occurring while the meal is passing along the small intestine. This allows for a more gradual introduction of glucose in to the body, and would give the other tissues more time to handle this nutrient.

The significance of the CCK effect on hexose absorption and on the overall plasma glucose concentration, including the indirect inhibition seen with delaying gastric emptying, could only be determined by a comprehensive *in vivo* study which would allow one to delineate the two effects.

Another prominent finding in this study was the decreased sensitivity to CCK in experimental diabetes. These results indicate that an altered CCK sensitivity is one of the many factors involved in the etiology of hyperglycemia found in these animals. It is evident that further experiments are needed to: 1) substantiate this finding, 2) determine if the locus of action is similar to that seen in the untreated (control) tissue, and 3) establish if the inhibition is mediated by a reduction in the abundance of SGLT1 as seen in animals not treated with streptozotocin, and 4) how much this defective pathway is contributing to hyperglycemia.

Regarding the changes in SGLT1 abundance, these results were critical to the understanding of the decrease in the rate of hexose observed, but require a substantial amount of follow-up. What should be done is first determine if there is a vesicular pool located in the enterocytes containing the SGLT1 transporter, possibly by using subcellular fractionation (Simpson, Yvers et al. 1983), next determine if any recycling exists using a cell surface biotinylation followed by high resolution 2D electrophoresis (Hare and Taylor 1992) and finally determine if the CCK-negative feedback pathway for hexose absorption is operating in the other direction by increasing SGLT1 proteins in the plasma membrane by other intestinal peptides such as GLP-2 (Cheeseman 1997).

The endothelin link with CCK was based on preliminary data and can only be substantiated by showing a CCK-sensitive secretion of endothelin by endothelial cells, and by isolating CCK A-type receptors on the endothelial cells.

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